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Letters to the Editors

EDWARD BROWNING MEIGS
1879-1940

Edward Browning Meigs died November 5, 1940. Born in Philadelphia, September 10, 1879, he represented the ninth generation of the Meigs family in this country. He graduated from Princeton University in 1900, and from the Medical School of the University of Pennsylvania in 1904. During 1904-06 he was assistant in physiology at the University of Pennsylvania; from 1907-10, instructor in physiology at Harvard Medical School; from 1910-15, fellow in physiology at the Wistar Institute, Philadelphia. During this time he worked at the Physiological Institute at the University of Jena, in the Physiological Laboratory, Cambridge, England, and the Marine Biological Laboratory at Woods Hole, Massachusetts.

In 1915, Dr. Meigs took charge of the newly organized laboratory at Beltsville, Maryland, for the study of the nutrition of dairy cows in the Dairy Division of the Bureau of Animal Industry, United States Department of Agriculture—later the Bureau of Dairy Industry. When this work was subsequently organized as the Division of Nutrition and Physiology in 1937, Dr. Meigs was made Chief of this Division.

Before entering the Government service Dr. Meigs had published twenty-five papers on the physiology of muscular contraction, the osmotic properties of muscle, and the osmotic properties of calcium and magnesium. His contribution to the nutrition and physiology of the dairy cow included twenty-seven papers and bulletins. In this field he made notable contributions to the knowledge of the mineral metabolism of the dairy cow and the part played by hay and other forages in supplying minerals and vitamins to milking cows. He was able to demonstrate, as the result of an outbreak of mastitis, that mastitis could be produced or cured almost at will by the control of physical conditions.

Dr. Meigs' great grandfather, grandfather, and father were

physicians. A description of his father, written in a memoir, was equally characteristic of Dr. Meigs. He characterized his father as having a "passion for the truth," "his mind had a peculiar quality of exactness; he never deluded himself into thinking that he understood a matter until he had thoroughly grasped it," and "it is difficult to say whether more of my father's energy was given to the practice of medicine or to scientific research." There was a further similarity in the father and son in their interest and study of the chemistry of milk and its formation. It was this interest, undoubtedly, that made his position with the Government so attractive and all absorbing.

Ill health interfered with his work in the past few years. He was obliged to give up the administrative work of his position. He continued, however, his active interest in the work of his Division and devoted himself to the preparation for publication of the results accumulated in his active years.

Those who were fortunate enough to know Dr. Meigs will remember his gentle courteous manners and unfailing generosity of thought and action, and his true friendliness, along with his scholarly attainments and zeal for the truth.

PAUL E. HOWE

CYSTINURIA. THE EFFECT OF VARIOUS AMINO ACIDS ON THE EXCRETION OF CYSTINE*

By W. C. HESS and M. X. SULLIVAN

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(Received for publication, April 10, 1941)

The condition known as cystinuria has long baffled investigators who have tried to explain its mechanism and etiology. The finding, repeatedly verified, that the level of cystine excretion by a cystinuric is not increased by the ingestion of cystine itself but is increased by feeding proteins, rich in sulfur, has always been difficult to understand. A new impetus was given to the study of cystinuria, however, by the investigations of Brand, Cahill, and Harris (1) who suggested that, "The cystine excretion in cystinuria is caused mainly by dietary methionine, but the inborn error of metabolism is concerned with the handling of *cysteine*." They consider that, "One of the pathways of methionine catabolism is its conversion into *cysteine*."

This hypothesis was proposed because the ingestion of 8.0 gm. of *dl*-methionine or 8.8 gm. of cysteine hydrochloride produced an increased excretion of 2.0 gm. of cystine in a cystinuric, while the ingestion of cystine produced no extra urinary cystine. Brand, Cahill, and Block (2) also found that, while the ingestion of 5.5 gm. of homocysteine produced 1.6 gm. of extra cystine in the cystinuric urine, the feeding of 7.2 gm. of homocystine produced no extra cystine.

Lewis, Brown, and White (3) fed *dl*-methionine and cysteine hydrochloride to a cystinuric and, while they found extra cystine in the urine, the amounts were considerably less than those reported by Brand, Cahill, and Harris (1). Andrews and Randall

* Preliminary reports of this work were presented before the meetings of the American Society of Biological Chemists held at Baltimore, March, 1938, and at New Orleans, March, 1940.

(4) administered *dl*-methionine to a cystinuric boy and were unable to detect any significant increase in urinary methionine. They suggested the possibility of variation among cystinuric individuals and also the desirability of studying as wide a range of subjects and conditions as possible. Later, Andrews, Andrews, and Rutenber (5) repeated their experiments upon the same boy, now postadolescent, and found that the ingestion of large doses of *dl*-methionine (up to 5.0 gm. daily) caused an increase in urinary cystine, a finding reaffirmed by Andrews and Andrews (6).

It may be pointed out that most students of cystinuria have estimated only the free cystine, that is the cystine which was in solution in the urine and that present in the sediment, and have ignored cystine present in a bound form. That the urinary cystine may be bound to a considerable degree was early suggested by Delépine (7). Later, Sullivan (8) reported that a cystine compound is present in normal urine and Brand, Harris, and Biloon (9) found that a cystinuric urine contained a cystine compound which gradually decomposes, with the liberation of free cystine.

Sullivan and Hess (10) using improved procedures then showed that normal urines contain a small amount of free cystine and in addition complexes which liberate cystine on standing and also on treatment with acids or alkali. Acid hydrolysis of urine gives, in fact, a considerable increase in the cystine content as determined colorimetrically both for the normal and the cystinuric cases.

Since we had the opportunity of studying several cystinuric individuals, attention was paid to the free cystine and the bound cystine and to the effect of the ingestion of various amino acids on the amount of cystine excreted. The results of these experiments are given in this paper.

EXPERIMENTAL

Subject M. K. was a 40 year-old male of Jewish parentage, 73 kilos in weight. There was a history of calculus formation as evidenced by renal colic and the passage of small cystine stones about 20 mm. in diameter. During the period of the present investigation the urine contained numerous cystine crystals but no stones.

Subject D. R. was a 38 year-old male of Jewish parentage, 67.5 kilos in weight. The subject also had a history of calculus

formation. His urine always contained a considerable number of cystine crystals. Both subjects were otherwise in excellent physical condition. Since they were working during the experimental periods, it was impossible to hospitalize them or to control their diets rigorously. However, both men were intelligent and coöperative and maintained a fairly strict medium protein dietary as was evidenced by the constancy of their cystine and sulfur output during the numerous control periods. During several experimental and control periods, Tables I and II, total urinary nitrogen and creatinine were determined. The constancy of these values served to confirm the fact that the subjects were living upon a rather regular dietary. It is evident that the regularity of the sulfur excretion, during the control periods, is as good a check upon the dietary intake as are the determinations of nitrogen and creatinine.

Subject W. H. was a 40 year-old male also of Jewish parentage, 78 kilos in weight. This subject was used as normal control and has never shown any indication of greatly increased cystine excretion. Similar precautions to keep the diet rather uniform were taken in his case as with the other two subjects. In both the cystinuric and normal cases the urine volume did not vary greatly from period to period.

Methods

The cystine determinations in the urine were made by means of the Sullivan (11) method and the procedures employed by Sullivan and Hess (10) for normal urines. The cystine in the sediment was determined by filtering the whole urine, dissolving the sediment in 0.1 N HCl, and analyzing an aliquot of this solution. The determinations of the cystine content before and after hydrolysis were all made on the filtered urines. The sulfur partition was made gravimetrically by the methods of Folin (total sulfate and ethereal sulfate) and Benedict (total sulfur).

The compounds administered were (*a*) *l*-methionine isolated from casein by the method of Hill and Robson (12) (the total sulfur showed it to be 99 per cent pure); (*b*) *l*-cysteine hydrochloride prepared from a highly purified sample of *l*-cystine by reduction by tin and hydrochloric acid and subsequent isolation of the crystalline compound; (*c*) *dl*-alanine, *d*-glutamic acid, and glycine

obtained from the Eastman Kodak Company; (*d*) glycylmethionine prepared from naturally occuring methionine by methods detailed by Hess and Sullivan (13). The total sulfur showed it to be 99.9 per cent pure.

The various compounds were administered by mouth, after breakfast, and were followed by ingestion of water. The urine specimens were collected over chloroform and the period of collection was from 8 a.m. to 8 a.m. The specimens were analyzed immediately after collection.

Results

In Table I are presented the data for a series of experiments upon subject M. K. As judged by comparison of the average daily output of the immediate pre- and postfeeding periods on the basal ration with the actual amount excreted during the various addition periods, the only change in diet which produced an increase in cystine excretion by M. K., Table I, is a high protein diet. It may be emphasized that neither the ingestion of cysteine hydrochloride nor of *l*-methionine caused any noticeable increase in the cystine excretion. It would appear then that subject M. K. fed different levels of methionine upon three separate occasions did not excrete extra urinary cystine following such feedings.

Finding our results with M. K. at variance with those of Brand, Cahill, and Harris (1), of Lewis, Brown, and White (3), and of Andrews and coworkers (5, 6), we conducted a similar series of experiments upon another definite cystinuric, D. R., with results more like those of other investigators. The results are given in Table II.

In the case of D. R. the ingestion of 2.0 gm. of *l*-methionine for 3 successive days (Period 2) produced during these 3 days and the next an excretion of 0.61 gm. of extra cystine. The total extra sulfur, in the urine, accounted for 87 per cent of the sulfur fed as methionine, and the extra cystine sulfur was 12.4 per cent of the sulfur fed. A repetition of this experiment is given in Table II (Period 22). The ingestion of 6.0 gm. of *l*-methionine during the 3 day period led to the excretion of 0.70 gm. of extra cystine. Since the sulfur output did not return to normal until the 3rd day following the last administration of methionine, a total of 5 days was included in the feeding period. It may be noted that the

cystine output of the control period following the ingestion of the methionine (Period 23) was no greater than that during the control period prior to the ingestion of the methionine (Period 21). The

TABLE I

Effect of Amino Acids on Excretion of Cystine in Cystinuria (Subject M. K.)

Period No.	Date	Substance fed	Amount	Cystine* Before hydrolysis	Cystine* After hydrolysis	Sulfur Total	Sulfur Total sulfate	Sulfur Neutral	Nitrogen
	1935		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1†	Jan. 17-20			0.62	0.86	0.74	0.41	0.33	
2	" 21	Methionine	2.0	0.68	0.88	0.99	0.68	0.31	
	" 22	"	2.0	0.60	0.69	0.81	0.53	0.28	
	" 23			0.61	0.94	1.03	0.66	0.37	
3†	" 24-25			0.51	0.80	0.68	0.36	0.32	
4	" 26	Cysteine HCl	2.6	0.54	0.82	0.88	0.58	0.32	
	" 27			0.55	0.74	0.92	0.51	0.41	
5†	" 28-Feb. 4			0.51	0.67	0.73	0.43	0.30	
6	Feb. 5	Glycine	10.0	0.41	0.65	0.80	0.51	0.29	
	" 6	"	10.0	0.59	0.74	0.80	0.50	0.30	
7†	" 9-11			0.53	0.70	0.71	0.41	0.30	
8	Mar. 8			0.36	0.72	0.75	0.43	0.32	8.9
9	" 10	High protein diet		0.48	1.01	1.00	0.69	0.31	16.9
	" 11	" " "		0.55	0.89	0.85	0.47	0.38	17.5
	" 12	" " "		0.72	0.82	0.95	0.55	0.40	17.2
	" 13			0.48	0.81	0.80	0.44	0.36	12.8
10†	" 14-15			0.45	0.72	0.81	0.55	0.36	11.2
11†	" 22-23			0.52	0.81	0.99	0.63	0.36	
12	" 24	Methionine	5.0	0.45	0.76	1.55	1.19	0.36	
	" 25	"	5.0	0.53	0.83	1.62	1.24	0.38	
13†	" 27-28			0.58	0.81	1.13	0.75	0.38	
	1940								
14†‡	Oct. 17-19			0.86	1.02	0.86	0.58	0.28	11.82
15	" 20	Methionine	3.0	0.83	1.03	0.97	0.67	0.30	12.49
	" 21	"	3.0	0.97	1.03	1.18	0.85	0.33	11.93
	" 22			0.78	0.95	1.24	0.89	0.35	11.69

* These values were obtained by adding the cystine of the respective sediment (20 to 160 mg.) to the cystine in the filtrate.

† In this period the figures indicate the average daily excretion.

‡ During Periods 14 and 15 daily creatinine determinations were made. The values varied from 1.59 to 1.68 gm. per day.

TABLE II

Effect of Amino Acids on Excretion of Cystine in Cystinuria (Subject D. R.)

Period No.	Date	Substance fed	Amount	Cystine*		Sulfur			Nitrogen
				Before hydrolysis	After hydrolysis	Total	Total sulfate	Neutral	
	1939		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1†	Oct. 31–Nov. 4			0.38	0.70	0.57	0.30	0.27	
2	Nov. 9	Methionine	2.0	0.74	0.76	0.79	0.43	0.36	
	" 10	"	2.0	0.83	1.02	1.01	0.58	0.43	
	" 11	"	2.0	0.84	1.04	1.09	0.64	0.45	
	" 12			0.79	0.81	0.97	0.61	0.36	
3	" 13			0.76	0.81	0.80	0.42	0.38	
4	" 16	Alanine	5.0	0.94	0.97	0.75	0.39	0.36	
	" 17	"	5.0	0.91	0.97	0.86	0.40	0.46	
	" 18			0.77	0.88	0.81	0.40	0.41	
5	" 19			0.58	0.71	0.70	0.37	0.33	
6†	Dec. 7–9			0.80	0.95	0.73	0.35	0.38	
7	" 11	Glutamic acid	5.0	0.87	0.99	0.72	0.30	0.42	
	" 12	" "	5.0	0.72	0.92	0.62	0.26	0.38	
	" 13			0.92	1.00	0.74	0.37	0.37	
8	" 14			0.72	0.85	0.71	0.35	0.36	
	1940								
9†	Feb. 19–20			0.85	0.95	0.88	0.51	0.37	
10	" 21	Glycine	5.0	0.86	1.03	0.90	0.50	0.40	
	" 22	"	5.0	1.00	1.05	0.99	0.56	0.43	
	" 23			0.90	0.94	0.95	0.53	0.42	
11	" 24			0.82	0.91	0.90	0.51	0.39	
12	" 25	Glycylmethionine	2.76	0.99	1.05	0.99	0.56	0.43	
	" 26	"	2.76	1.14	1.19	1.14	0.69	0.45	
	" 27	"	2.76	0.90	1.01	1.04	0.58	0.46	
	" 28			1.00	1.17	1.16	0.68	0.48	
13	" 29			0.80	0.89	0.89	0.52	0.37	
14†	June 28–30			0.55	0.65	0.90	0.41	0.49	
15	July 1	Cysteine HCl	2.6	1.14	1.25	1.10	0.51	0.59	
	" 2			0.67	0.75	1.01	0.49	0.52	
16	" 3			0.74	0.81	0.84	0.38	0.46	
17	" 4	Cystine	2.0	0.60	0.80	1.07	0.66	0.41	
	" 5			0.65	0.79	1.03	0.62	0.41	
18	" 6			0.69	0.76	0.88	0.45	0.43	

TABLE II—*Concluded*

Period No.	Date	Substance fed	Amount	Cystine*		Sulfur			Nitrogen
				Before hydrolysis	After hydrolysis	Total	Total sulfate	Neutral	
	1940		gm.	gm	gm	gm	gm	gm	gm
19†‡	Nov. 8–12			0.91	0.99	0.89	0.41	0.48	9.53
20	" 13	Alanine	5.0	1.11	1.14	0.91	0.35	0.56	9.26
	" 14	"	5.0	1.05	1.11	0.90	0.38	0.52	9.49
	" 15			0.88	1.13	0.98	0.45	0.53	9.60
21	" 16			0.80	0.91	0.94	0.45	0.49	9.48
22	" 17	Methionine	2.0	0.72	0.96	1.02	0.45	0.57	10.40
	" 18	"	2.0	0.84	1.25	1.11	0.54	0.57	10.80
	" 19	"	2.0	0.85	1.14	1.26	0.70	0.56	10.66
	" 20			0.94	1.00	1.15	0.60	0.55	10.88
	" 21			0.80	0.95	1.16	0.64	0.52	10.52
23	" 22			0.78	0.90	0.92	0.48	0.44	10.73
24	" 23	Glycylmethionine	2.76	0.86	1.09	1.02	0.51	0.51	10.56
	" 24	"	2.76	0.88	1.12	1.17	0.63	0.54	10.55
	" 25	"	2.76	0.93	1.12	1.18	0.61	0.57	10.93
	" 26			0.84	1.06	1.09	0.57	0.52	10.76
25	" 27			0.74	0.92	0.94	0.51	0.43	10.76

* These values were obtained by adding the cystine of the respective sediment (70 to 460 mg.) to the cystine in the filtrate.

† In this period the figures indicate the average daily excretion.

‡ In Periods 19 to 25 inclusive daily creatinine determinations were made. The values varied from 1.89 to 2.43 gm. per day.

extra sulfur excreted during the 5 days accounted for 81.4 per cent of the sulfur fed as methionine.

Subject D. R. apparently does excrete extra cystine after the feeding of methionine, thus differing from subject M. K. In the experiments of Brand, Cahill, and Harris (1) the ingestion of 4.0 and 8.0 gm. of *dl*-methionine caused an excretion of 0.63 and 2.03 gm. of extra cystine respectively. These values especially after the ingestion of 8.0 gm. of *dl*-methionine are greater than those obtained upon subject D. R. In a similar experiment Lewis, Brown, and White (3) fed 4.7 gm. of *dl*-methionine and found 0.56 gm. of extra cystine in the urine, a value closely approximating those obtained by us on subject D. R.

Schmidt, Allen, and Tarver (14) have suggested that the con-

version of methionine to cystine may take place when the methionine is contained in a peptide. With this hypothesis in mind glycylmethionine was synthesized (Hess and Sullivan (13)) and fed to subject D. R. After the ingestion of 8.28 gm. of glycylmethionine, equivalent to 6.0 gm. of methionine (Period 12, Table II), there was excreted 0.82 gm. of extra cystine. The total extra sulfur excreted was 58 per cent of the sulfur fed. The repetition of the ingestion of glycylmethionine (Period 24, Table II) showed the same phenomena as the first experiment. The feeding of 8.28 gm. of glycylmethionine led to an excretion of 0.75 gm. of extra cystine and an excretion of 57.3 per cent of the sulfur fed. Both of these experiments indicate, perhaps, poorer absorption of the peptide than of methionine itself. However, of the extra sulfur excreted in the first experiment, 29 per cent was cystine sulfur as compared with 14 per cent when 6.0 gm. of methionine were fed, and in the second experiment 27 per cent of the extra sulfur excreted was cystine sulfur as compared with 17.7 per cent when 6.0 gm. of methionine were fed. Thus, based on the extra urinary sulfur, there is some slight evidence that this particular methionine peptide is a better source of extra urinary cystine than is methionine itself.

Since the administration of methionine produced each time a definite increase in the output of cystine in the urine of subject D. R., it was considered worth while to test some other amino acids as to their effect on the cystine output. 5 gm. of alanine were fed for 2 successive days (Period 4, Table II). As judged by periods 3, 4, and 5 (Table II), the urine showed 0.54 gm. of extra cystine for the 2 feeding days and the following day. In other words the ingestion of 10 gm. of alanine produced 87 per cent as much extra cystine as did the ingestion of 6.0 gm. of *l*-methionine. The total sulfur output of the urine was likewise increased and the extra cystine sulfur accounted for 85 per cent of the extra total sulfur. 5 gm. of glycine were than fed for 2 successive days (Period 10) and as a result of the ingestion of 10 gm. of this amino acid 0.23 gm. of extra cystine was excreted, as determined by a comparison of Period 10 with Periods 9 and 11 (Table II). The extra total sulfur output was 0.17 gm., exactly the same as after feeding 10 gm. of alanine. The ingestion of 10 gm. of alanine was repeated at a subsequent time following a 5 day control period

(Periods 19 and 20) and resulted in the excretion of 0.48 gm. of extra cystine. This value is but slightly less than the 0.51 gm. excreted previously following the administration of the same amount of alanine (Period 4, Table II). After these findings with subject D. R. the data for the feeding of 20 gm. of glycine to subject M. K. (Period 6, Table I) were reconsidered and while only 0.02 gm. of extra cystine was excreted the extra total sulfur output was 0.16 gm., practically the same as with subject D. R. following the ingestion, in separate experiments, of 10 gm. each of alanine and glycine.

The ingestion of 5.0 gm. of glutamic acid for 2 successive days (Period 7, Table II) resulted in the excretion of 0.21 gm. of extra cystine, approximately the same increase as after feeding a similar amount of glycine. However, after the feeding of the glutamic acid there was a slight diminution of the total sulfur output instead of the increase which followed the administration of alanine and glycine. Luck and Amsden (15), working with dogs, found that parenterally administered amino acids stimulated endogenous protein catabolism and caused a 30 to 40 per cent increase in the excretion of total sulfates. Reid (16) reported that alanine and especially glycine increased the excretion of inorganic sulfur when fed to fasting dogs. Our findings that amino acids given by mouth stimulate endogenous protein catabolism and increase sulfur excretion agree with those of Luck and Amsden and of Reid.

In the case of subject D. R. the ingestion of 2.6 gm. of cysteine hydrochloride produced a marked increase in the extra urinary cystine (Period 15, Table II). As judged by Periods 14, 15, and 16, Table II, the extra cystine excreted accounted for 27 per cent of the sulfur ingested, a value far in excess of those for either methionine or glycylmethionine which caused an extra excretion of cystine corresponding to 12.4 and 17 per cent of the sulfur fed.

In order to complete the series of the sulfur-containing amino acids fed, 2.0 gm. of cystine were administered (Period 17, Table II). In agreement with the findings of all other investigators we found no extra urinary cystine was produced. There was a slight decrease in the neutral sulfur excreted and 86.9 per cent of the sulfur ingested as cystine was excreted as inorganic sulfate sulfur.

The two cystinurics, M. K. and D. R., differ markedly in the

response to the addition to their diets of methionine, cysteine, and glycine. M. K. excreted no extra cystine, while D. R. showed extra cystine after each of these amino acids.

Since the cystinurics investigated differed markedly as regards the effect of methionine, etc., on cystine excretion, recourse was had to a normal subject in whom urinary cystine excretion tended to be somewhat higher than in other normals studied by us. Mueller (17) when feeding small amounts of methionine to an adult male found that the methionine sulfur was rapidly oxidized. Medes (18) reported that the sulfur of *l*-methionine was recovered 100 per cent in the urine as inorganic sulfate within 16 hours after ingestion.

Accordingly, as detailed in Table III, a normal subject, W. H., was fed 5.0 gm. of glycine (Periods 2 and 18), glutamic acid (Period 4), and alanine (Periods 6 and 16) for 2 successive days. A study of Table III shows that none of the amino acids caused a noticeable increase in cystine excretion. The ingestion of 10 gm. of alanine in two separate experiments, however, caused an increased sulfur output of 0.23 and 0.36 gm., while glycine had little effect upon sulfur excretion.

The normal cystine output of this subject, though somewhat higher than that of other normals of this laboratory, was quite uniform, as can be noted from the data presented in Table III for the various control periods (Nos. 1, 3, 5, 7, etc.). The uniformity of the diet can be judged from the regularity of the nitrogen and creatinine excretion as well as the total sulfur.

Virtue and Lewis (19) have reported the excretion of some substance containing the disulfide grouping, other than cystine, following the ingestion of methionine by rabbits. We have looked for such a substance in the urine of W. H. following the administration of methionine. By the use of the Okuda method as detailed previously (Sullivan and Hess (10)) it is possible to distinguish between cystine and other disulfides. The hydrolysates of the urine of W. H. before and after feeding methionine gave practically the same value by the Okuda method as they did by the Sullivan method. Apparently, in this urine with relatively much smaller dosage of methionine than was used by Virtue and Lewis (19) there is no evidence for the excretion of any disulfide compound other than cystine, normally present in small amounts.

TABLE III

Effect of Amino Acids on Excretion of Cystine in a Normal (Subject W. H.)

Period No.	Date	Substance fed	Amount	Cystine		Sulfur			Nitrogen
				Before hydrolysis	After hydrolysis	Total	Total sulfate	Neutral	
	1940		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1*	Jan. 15–18			0.11	0.17	0.86	0.65	0.21	
2	" 19	Glycine	5.0	0.08	0.15	0.89	0.72	0.17	
	" 20	"	5.0	0.10	0.14	0.86	0.69	0.17	
	" 21			0.10	0.15	0.79	0.61	0.18	
3	" 23			0.08	0.15	0.80	0.63	0.17	
4	" 24	Glutamic acid	5.0	0.09	0.15	0.79	0.59	0.20	
	" 25	" "	5.0	0.10	0.14	0.88	0.68	0.20	
	" 26			0.10	0.14	0.85	0.65	0.20	
5	" 27			0.10	0.14	0.84	0.67	0.17	
6	" 28	*dl*-Alanine	5.0	0.09	0.13	0.89	0.76	0.13	
	" 29	"	5.0	0.08	0.12	0.97	0.81	0.16	
	" 30			0.10	0.14	0.86	0.63	0.23	
7	" 31			0.08	0.12	0.82	0.60	0.22	
8*	July 6–7			0.05	0.14	0.83	0.67	0.16	
9	" 8	Cysteine HCl	2.6	0.07	0.16	1.24	1.08	0.16	
	" 9			0.05	0.15	0.86	0.69	0.17	
10	" 10			0.05	0.16	0.85	0.68	0.17	
11	" 11	*l*-Methionine	2.0	0.04	0.18	1.18	0.89	0.29	
	" 12			0.05	0.15	0.84	0.68	0.16	
12.	" 13			0.05	0.16	0.83	0.66	0.17	
13	" 14	Cystine	2.0	0.06	0.16	1.31	1.12	0.19	
	" 15			0.05	0.16	0.85	0.67	0.18	
14	" 16			0.05	0.14	0.86	0.71	0.15	
15*†	Nov. 24–27			0.06	0.14	0.72	0.54	0.18	9.01
16	" 28	*dl*-Alanine	5.0	0.07	0.17	0.73	0.57	0.16	9.72
	" 29	"	5.0	0.07	0.19	0.87	0.66	0.21	10.61
	" 30			0.07	0.18	0.94	0.76	0.18	9.45
17	Dec. 1			0.06	0.14	0.74	0.58	0.16	9.22
18	" 2	Glycine	5.0	0.07	0.17	0.74	0.55	0.19	9.58
	" 3	"	5.0	0.06	0.19	0.76	0.55	0.21	9.44
	" 4			0.06	0.15	0.75	0.58	0.17	9.36
19	" 5			0.06	0.15	0.76	0.60	0.16	9.33

* In this period the figures indicate the average daily excretion.

† In Periods 15 to 19 inclusive daily creatinine determinations were made. The values varied from 1.62 to 1.88 gm. per day.

SUMMARY

The urine of one cystinuric, M. K., showed no increase in cystine content following the ingestion of from 4 to 10 gm. of *l*-methionine upon three separate occasions and a very slight increase following the ingestion of cysteine hydrochloride. On the other hand, an increased protein intake markedly increased the cystine output. The ingestion of large amounts of glycine was without effect upon the cystine excretion but did increase, somewhat, the total sulfur excretion.

The urine of another cystinuric subject, D. R., gave results in rather marked contrast to that of M. K. The ingestion of *l*-methionine and of glycylmethionine upon several occasions and also the feeding of cysteine hydrochloride increased the cystine content of the urine. In fact the cysteine hydrochloride gave a greater increase of extra cystine than did methionine or glycylmethionine. However, the ingestion of three non-sulfur-containing amino acids, alanine, glycine, and glutamic acid, also produced an increase in cystine excretion. The action of the alanine upon two separate occasions was much more marked than that of either glycine or glutamic acid and approximated the stimulatory power of methionine. The ingestion of the glycine and the glutamic acid, in the same amounts as the alanine, produced slightly less than half as much extra cystine. As has been found repeatedly, cystine ingested was rather completely oxidized and yielded no extra urinary cystine.

In the urine of the normal subject, W. H., the cystine excretion was within the normal limits following the ingestion of glycine, alanine, glutamic acid, *l*-methionine, cysteine hydrochloride, or cystine. However, the feeding of alanine, upon separate occasions, stimulated to a small degree the excretion of total sulfur as inorganic sulfate sulfur. In the normal subject the sulfur of the sulfur-containing amino acids was excreted mostly as inorganic sulfate sulfur. Following the ingestion of 2.0 gm. of methionine no evidence was found for the excretion of a disulfide compound other than the cystine normally present in small amounts.

In the case in which methionine increased cystine excretion, alanine did also and so did glycine and glutamic acid but to a lesser degree. In the normal individual, while the ingestion of the

amino acids led in no case to an increased excretion of cystine, alanine did bring about a slightly increased sulfur output. In the amino acid feeding the extra cystine excretion, when present, may be due in a large measure to a stimulation of metabolism.

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FAT METABOLISM AFTER LIVER INJURY*

FATTY ACID UTILIZATION BY RATS TREATED WITH CARBON TETRACHLORIDE ON DIETS WHICH WERE FAT-FREE OR CONTAINED FATS WITH HIGH OR LOW IODINE NUMBERS

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It has been reported (1) that carbon tetrachloride-treated albino rats show a decreased loss, an increased excretion, and an increased amount of body fatty acids when compared to normal controls. These results were interpreted as evidence that liver damage decreases fatty acid utilization. Utilization is taken to mean the oxidation of exogenous or endogenous fatty acids or their transformation to other substances; *i.e.*, that they disappear as such and cannot be accounted for in balance experiments. While the data in previous experiments were considered to indicate decreased fatty acid utilization in carbon tetrachloride-poisoned rats, an increased synthesis of fatty acids with no decrease in oxidation would account for the larger amount of fat recovered. To test the hypothesis that decreased utilization is the correct explanation, the effect of varying the level of fat intake was studied in balance experiments by using a fat-free diet. Similar balance experiments were conducted with a diet containing a highly unsaturated fat (cod liver oil). This was done to investigate the suggestion made earlier (1) that the observed decrease in fatty acid loss is due to the interference with desaturation mechanisms shown to follow liver damage (2).

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Procedure

Three groups of thirty-six male albino rats[1] were used. Each of these groups was subdivided into three divisions of twelve animals each, one of which was used to determine the fatty acid content of the animals at the start of the experimental period and which was sacrificed at the time the other two were placed on the diet to be studied. Of these latter two divisions, one served as a normal control, while the members of the other received 0.05 cc. of carbon tetrachloride subcutaneously every other day

TABLE I

Composition of Fat-Free Ration

Sucrose	12.0 kilos
Casein, fat-free*	3.2 "
Salt mixture (4)	0.8 kilo
$NaCl$	5.0%
$CaCO_3$	36.0%
$CaCl_2 \cdot 6H_2O$	28.4%
$CaHPO_4 \cdot 2H_2O$	21.8%
$FeCl_3 \cdot 6H_2O$	2.4%
Linoleic acid	75 gm.
Calciferol†	12 mg.
β-Carotene	100 "
Thiamine chloride‡	100 "
Calcium pantothenate‡	160 "
Pyridoxine hydrochloride‡	160 "
Riboflavin‡	100 "

* Labco vitamin-free casein, Casein Company of America, Inc., New York. Ether-soluble material, 0.84 per cent.

† Generously supplied by the Abbott Laboratories, North Chicago.

‡ Generously supplied by Merck and Company, Inc., Rahway.

during the period of observation. In order to insure the comparability of the results, the use of litter mates was adopted. That is, each group of thirty-six animals consisted of twelve sets of litter mates, one litter mate from each set being placed in each division.

The amount of food eaten daily by the treated animals was measured. A paired feeding technique was followed, the food intake of each normal rat being restricted to that of its treated

[1] 80 to 100 gm., Sprague-Dawley, Inc., Madison.

litter mate. The experimental period was 2 weeks for each group.

The three diets used were developed from the fat-free ration of Quackenbush *et al.* (3) (Table I).

Group I was given a diet made by incorporating 7 per cent ethyl stearate into one-third of the fat-free ration (Table I). The results with this group were used to confirm the earlier observations, which were not made on litter mates fed the synthetic diet. Group II was given the fat-free diet alone, while Group III received the fat-free diet plus 7 per cent cod liver oil. The actual fatty acid content of the three diets is as follows: Group I, stearate, 8.61 per cent; Group II, fat-free, 0.27 per cent; Group III, cod liver oil, 6.42 per cent.

Methods

The methods of analysis used have been described earlier (1). The animals were kept in individual glass cages and at the end of the experimental period were killed. After uneaten food had been removed and weighed, the fatty acids of the body of the animal, feces, and spilled food were dissolved in boiling alcoholic potassium hydroxide, filtered through glass wool, and made to volume (1000 cc.) with alcohol. Aliquots were dealcoholized under a vacuum, acidified, and extracted overnight with petroleum ether in continuous liquid-liquid extractors. The petroleum ether solution was evaporated to a volume of 10 to 20 cc., washed through filter paper with petroleum ether into tared weighing flasks, and weighed after all solvent had been removed *in vacuo*. Because of the difficulty in obtaining digitonin the amount of fatty acids present in the extract was not determined by subtracting the weight of sterol present, as was done in the earlier work. Instead the weighed fatty material was dissolved in alcohol and titrated hot with 0.1 N sodium hydroxide, with phenolphthalein as an indicator. The fatty acids found were calculated by multiplying the cc. of alkali used by an arbitrary factor, 0.273, derived from the molecular weights of stearic, palmitic, and oleic acids. The results obtained by this method compared favorably with those found by precipitating sterol as digitonide and calculating the fatty acids by difference, when "fatty acid" values alone were considered. The values for the smaller sterol component of the extract were not considered to be as accurate

of the normal and treated animals receiving the cod liver oil diet was observed, this difference was very much less than that found when the stearate diet was used. The difference in fatty acid utilization of the treated animals on these two diets was greater than that between treated and normal animals on the cod liver oil diet (0.89 and 0.67 gm., respectively). The fatty acid utilization, over the 2 week period, of the normal animals of both groups and the treated animals of the cod liver oil group are roughly equivalent (4.54, 4.21, and 3.87 gm.), while that of the treated animals receiving the saturated fat was of a definitely lower order (2.98 gm.). The larger amount of fatty acids in the bodies of the animals fed cod liver oil at the start of the period and their greater fat intake (as compared to the animals fed stearate) may have played a part in the increased utilization found for both normal and treated animals of this group. Since the normal rats receiving the cod liver oil diet showed an increase in fatty acid utilization of 8 per cent, while that of the treated rats on this diet increased 30 per cent over comparable animals ingesting the stearate ration, such an influence must not have been an important factor in producing the differences noted in the fatty acid utilization of the treated animals of these two groups. It seems apparent that the fatty acids of cod liver oil are either more readily converted by the liver into types suitable for utilization by other tissues, or do not require a preliminary transformation in the liver. It is believed that the high degree of unsaturation possessed by the average fatty acid found in cod liver oil is responsible, since the stearate diet was in all other respects complete. It should be noted here that the essential fatty acid, linoleic acid, was present in adequate amounts in the stearate diet; therefore the presence in abundance of other unsaturated acids in the cod liver oil must have been the determining factor. This concept is supported by considering the per cent reduction in fatty acid utilization brought about by carbon tetrachloride treatment in this and earlier work (1). In the previous report it was shown that liver damage reduced fatty acid utilization to 86 per cent of the normal value when the diet contained a preponderance of saturated fatty acids (coconut oil) but also a relative abundance of unsaturated acids (basal

diet, Purina dog chow). In the present report it is seen that where abundant unsaturated fatty acids are available (cod liver oil diet), and therefore similar conditions to those described above exist, the utilization is again reduced to 86 per cent of the normal. When, however, the unsaturated fatty acid content of the diet is limited to essential quantities of linoleic acid, the fatty acid utilization is reduced over twice as much; in this case to 70 per cent of the normal value.

Since the unsaturated fatty acid pattern found in cod liver oil cannot be expected to fit exactly that demanded by the tissues of the rat, the residual difference between the fatty acid utilization of the normal and treated rats of the cod liver oil group is not unexpected. It is of course possible, if not probable, that other rearrangements than desaturation of the fatty acid molecule performed by the liver may play a part in the decreased utilization following liver injury.

SUMMARY

1. Fatty acid balance experiments were conducted on three groups of male rats treated with carbon tetrachloride and litter mate normal controls. Three similar synthetic diets were used, one containing 7 per cent stearic acid, another 7 per cent cod liver oil fatty acids, and the third fat-free.

2. The fatty acid loss, or utilization, of the animals receiving the fat-free diet was a small fraction of that of the animals ingesting larger quantities of fat. There was no significant difference in fatty acid utilization between the normal and treated members of the fat-free group.

3. The fatty acid utilization of the carbon tetrachloride-treated animals receiving the stearate diet was much reduced below that of their normal litter mate controls. When cod liver oil replaced stearic acid in the diet, the difference between normal and treated animals was definitely decreased.

4. It is concluded that carbon tetrachloride poisoning does not increase fatty acid synthesis, and that the lack of abundant quantities of non-essential unsaturated fatty acids is a prime factor in the decreased utilization of fatty acids shown to follow liver damage.

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TRANSFORMATIONS OF PHOSPHORUS DURING GLUCOSE FERMENTATION BY LIVING CELLS OF STREPTOCOCCUS FAECALIS

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Considerable evidence exists for a phosphorylating glycolysis by preparations of bacteria (1–3), but little evidence is available on the intact living cell. Wiggert and Werkman (4) have shown the esterification of the cell inorganic phosphorus during glucose fermentation by *Aerobacter indologenes* and Macfarlane (5) has shown small uptakes of cell phosphate during glucose fermentation by *Bacterium coli*. In neither case was there an exchange of phosphorus between the cell and the suspending medium. Virtanen and Tikka (1) demonstrated the formation of hexose monophosphate and an unknown phosphate ester by *dried* cells of *Bacterium casei* ϵ, but were unable to show such a reaction in the living cells. Evidence is presented to show that during glucose fermentation by resting cells of *Streptococcus faecalis* inorganic phosphorus from within the cell and from the surrounding medium is converted into organic phosphorus compounds.

EXPERIMENTAL

Strain 10C-1 of *Streptococcus faecalis* from the Cornell University collection was used. This strain converts 98 per cent of the glucose fermented into lactic acid. 10 liters of 1 per cent tryptone, 1 per cent yeast extract, 0.8 per cent K_2HPO_4, and 0.2 per cent glucose were inoculated with 200 ml. of an 8 hour culture of the organism. After 15 hours incubation at 37°, the cells were harvested with a Sharples centrifuge, washed once with 0.9 per cent NaCl, and resuspended in 0.9 per cent NaCl, so that 10 ml. contained 1 gm. of the wet cells. Glucose, saline, or phosphate was added as indicated, and a little brom-thymol blue. The lactic

acid produced was titrated as formed with N NaOH. At indicated intervals samples were removed, centrifuged, and both the supernatant and the cells analyzed as indicated below.

1 gm. of the cells was immediately treated with 1 ml. of acetone (to aid in their disruption) and extracted with 25 ml. of 5 per cent trichloroacetic acid for 20 hours at room temperature. The cell débris was centrifuged off and the supernatant fluid fractionated with barium according to the method of Eggleton and Eggleton (6). The fractions were analyzed for inorganic phosphorus by the method of Fiske and Subbarow (7). Total phosphorus was determined as inorganic phosphorus after digestion with H_2SO_4 and H_2O_2. The barium-insoluble fraction was analyzed for hexose diphosphate by Roe's modification of the Seliwanoff method (8), for readily hydrolyzable organic phosphorus by heating at 100° for 7 minutes in N H_2SO_4 (presumably a measure of adenosine triphosphate), and for difficultly hydrolyzable organic phosphorus (not hydrolyzed within 180 minutes at 100° in N H_2SO_4, presumably the phosphoglyceric acids). The barium-soluble fraction was precipitated with alcohol according to the method of Cori and Cori (9). Organic phosphorus and the reduction value were determined on this precipitate (10).

Previous studies on the phosphorus transformations during glucose fermentation by intact bacterial cells have been concerned only with the acid-extractable portion. We have frequently found more inorganic phosphate utilized during glucose fermentation than could be accounted for by the organic phosphorus obtained in the extracts. We have therefore attempted to account for all of the phosphorus in the cell and the surrounding medium in the following manner. The first 20 hour extract was prepared and fractionated as described above. The cell débris was extracted for another 20 hour period under the same conditions. This extract yielded organic phosphorus which was all barium-soluble. A third extraction under the same conditions yielded no phosphorus. The phosphorus remaining in the cells after the third extraction may be regarded as acid-insoluble.

Results

Two types of experiments are described: one in which no phosphorus is added to the suspending medium and another in which adequate inorganic phosphorus is available.

Without Added Phosphorus—The data of such an experiment are given in Table I. The organisms were suspended in 0.9 per cent NaCl and divided into two portions. To one glucose was added to give a final concentration of 2 per cent, while the other received an equivalent quantity of water. Data on the phosphorus changes in the saline and in the trichloroacetic acid extract are recorded. Initially there is a small amount of inorganic

TABLE I

Phosphorus Changes during Glucose Fermentation by Streptococcus faecalis in Absence of Added Phosphorus

All data in Columns 2 through 9 are expressed as micrograms of phosphorus per gm. (wet weight) of bacterial cells. Column 1 represents the ml. of normal alkali required to neutralize the acid produced by 1 gm. of cells. Glucose fermentation was complete in 3 hours and thereafter no alkali was required. The data in Column 10 are expressed as per cent of Column 5 and are obtained from the sum of Columns 6 through 9.

	Acid produced	P in saline		P in trichloroacetic acid extract		Composition of organic P				
						Barium-insoluble			Barium-soluble	Total accounted for
		Inorganic	Organic	Inorganic	Organic	Δ_7	$\Delta_{180} - \Delta_7$	Resistant		
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Start		56	12	680	948	5	5	398	500	97
With 2% glucose after										
30 min. . . .	0.5	0	92	182	1280	110	15	190	1005	101
60 "	1.1	0	142	207	1342	107	3	207	1005	99
9 hrs.	1.6	68	192	610	650	57	50	90	608	124
Without glucose										
30 min. . . .	0.1	68	24	600	880	5	100	210	520	92
60 "	0.1	74	68	602	855				515	
9 hrs.	0.1	131	101	618	333	5	55	60	212	100

phosphate in the saline in which the cells are suspended. Without sugar both the inorganic and the organic phosphorus increase in the medium, while there is a decrease in the organic phosphorus of the cells. The difficultly hydrolyzable phosphorus (Column 7) tends to increase but there is little change evident in the other fractions studied. With glucose, however, inorganic phosphorus is removed from the saline (Column 2). Within the cell, inorganic phosphorus (Column 4) is converted into organic forms (Column

5) and appears principally as barium-soluble alcohol-insoluble material (Column 9). This material is not hexose monophosphate, since the reducing value by Folin's method is only 10 per cent that of glucose. The readily hydrolyzable organic phosphorus (Column

TABLE II

Phosphorus Changes during Glucose Fermentation by Streptococcus faecalis in Presence of Adequate Phosphorus

Column 1 represents ml. of normal alkali required to neutralize the acid produced by 1 gm. of bacterial cells. Data in Columns 2 through 10 are expressed as micrograms of phosphorus per gm. of cells. Glucose fermentation was complete at 150 minutes and thereafter no acid was produced. The data in Column 11 are the sum of Columns 2 through 10 with the omission of Column 5 and thus include the errors occurring in the phosphorus distribution. Data in Column 12 are obtained from Column 11 and represent the per cent of the starting value.

	Acid produced	P in saline		Acid-soluble P						Acid-insoluble	Total recovered	Recovery
				First extract					Second extract			
						Organic P						
		Inorganic	Organic	Inorganic	Organic	Barium-insoluble		Barium-soluble				
						Δ_7	Rest					
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
Start		1208	342	1680	1590	30	650	1040	3380	1405	9,735	
With 2% glucose after												
30 min.	0.42	770	580	675	2475	125	703	1980	3250	1620	9,703	99
60 "	0.77	650	530	1020	2220	225	735	1500	3960	2120	10,740	110
150 "	1.62	1072	678	1160	2000	105	583	881	3130	1090	8,699	90
13 hrs.	1.62	1690	1010	1360	1760	90	693	1180	2280	1180	9,483	98
Without glucose												
30 min.	0.1	1250	370	1390	1640	85	575	1270	3350	1420	9,710	99
13 hrs.	0.1	1680	790	1190	1270	50	620	1410	3360	1255	9,355	96

6) increases, while the resistant phosphorus (Column 8) decreases. No hexose diphosphate could be detected by the Seliwanoff reaction. It is further apparent from the data that more inorganic phosphate has disappeared from the cell and the saline than can be accounted for by the organic phosphorus extracted.

With Added Phosphorus—Data from an experiment in which

1000 γ of inorganic phosphorus were added per gm. of bacterial cells are given in Table II. Attempts were made to account for all of the phosphorus involved by the methods previously described. It may be noted that, in general, the recoveries of phosphorus were satisfactory (Columns 11 and 12). In the absence of glucose there is little change in any of the fractions over the entire 13 hour period. With glucose, however, marked changes are evident. There is a decrease in the inorganic phosphorus in the saline (Column 2) and in the cell (Column 4) and a parallel increase in organic phosphorus (Column 5), which appears primarily in the barium-soluble alcohol-insoluble fraction (Column 8). The barium-insoluble material tends to increase somewhat more slowly than the barium-soluble fraction but an increase is evident (Column 7). The readily hydrolyzable phosphorus (Column 6) increases as well. In the presence of adequate quantities of inorganic phosphate, the level of organic phosphorus within the cell is increased above that which is evident without added phosphorus. As was indicated by the experiment in Table I, there seems to be a conversion of some of the inorganic phosphorus into acid-insoluble forms (Column 10) during the early stages of glucose fermentation.

DISCUSSION

The data presented in this paper may be interpreted in accordance with the phosphorylating glycolysis concept of intermediary metabolism, since there is an initial conversion of inorganic phosphorus into compounds resembling the hexose monophosphates and an increase in the adenosine triphosphate fraction during glucose fermentation. Although the compounds formed are not identical with those formed by yeast and animal tissues, the over-all changes in phosphorus agree with the theories of phosphorylating glycolysis. A further difference from yeast (5, 11) is that a phosphorus exchange occurs between the cell and the surrounding medium.

SUMMARY

At the beginning of glucose fermentation by resting cells of *Streptococcus faecalis* there is a marked decrease in inorganic phosphorus both in the cell and in the medium. The phosphorus

appears chiefly in the barium-soluble alcohol-insoluble fraction which has not yet been completely characterized. The changes in phosphorus distribution during glucose fermentation by the living cell are in accordance with the theories of phosphorylating glycolysis.

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FIXATION OF CARBON DIOXIDE BY PIGEON LIVER IN THE DISSIMILATION OF PYRUVIC ACID*

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It is now generally accepted that carbon dioxide is not the inert molecule in the physiology of the heterotrophic cell it was formerly thought to be, but plays an important rôle as a reactant in the metabolism of a variety of organisms (*cf.* Slade *et al.* (21), Werkman and Wood (26), Foster *et al.* (8), Krebs (14)).

Considerable evidence is now available (28, 29)[1] in the case of a number of bacteria that the initial reaction in fixation of carbon dioxide may be

$$(1) \qquad CO_2 + CH_3{\cdot}CO{\cdot}COOH = COOH{\cdot}CH_2{\cdot}CO{\cdot}COOH$$

The resulting oxalacetate is converted to other 4-carbon dicarboxylic acids. The question arises as to whether or not animal tissues fix carbon dioxide by this reaction. Attention was focused on this problem, particularly by Evans (4), who found that pyruvate is oxidized by pigeon liver even in the presence of malonate and is converted to 4-carbon dicarboxylic acids, α-ketoglutaric acid, and carbon dioxide. The only malonate-insensitive reaction that had been described in the literature for the synthesis of 4-carbon dicarboxylic acids from pyruvate was the Wood and Werkman reaction (27, 28) studied in connection with carbon dioxide fixation by the propionic acid bacteria. It was therefore evident that this reaction might occur in the dissimilation of pyruvate by pigeon liver, and be the source of 4-

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[1] Krampitz, L. O., Wood, H. G., and Werkman, C. H., unpublished data.

carbon dicarboxylic acids essential in the Krebs cycle. Evans and Slotin (6), Krebs and Eggleston (16), and Wood *et al.* (30) independently investigated the problem and each has presented some evidence favoring the suggestion that carbon dioxide is fixed by pigeon liver as pictured in Equation 1. The present investigation has been designed to examine this question in greater detail.

EXPERIMENTAL

The procedure consisted in dissimilating pyruvate by ground pigeon liver in a medium containing $NaHCO_3$ enriched with C^{13} (essentially the procedure of Evans and Slotin (6) and Krebs and Eggleston (16)). The products were fractionated, converted to carbon dioxide, and the C^{13} content determined with the mass spectrometer (17). A compound contains fixed carbon dioxide when the content of C^{13} is greater than the normal complement of C^{13}; *i.e.*, 1.09 per cent. Previous papers of the authors (28, 29) should be consulted for a more detailed account of the methods and calculations.

The distribution of fixed carbon within the products of the aerobic dissimilation of pyruvate with malonate as an inhibitor was studied first. The reaction mixture contained 24 gm. of liver which had been removed from pigeons immediately after decapitation and ground through an Apolant mincer into 166 ml. of ice-cold salt solution plus 15 ml. of 0.35 M $NaHCO_3$ containing 9.61 per cent C^{13}. The salt solution contained 9.0 gm. of NaCl, 0.46 gm. of KCl, 0.188 gm. of $MgSO_4$, 7.34 gm. of $Na_2HPO_4 \cdot 12H_2O$, and 0.62 gm. of $NaH_2PO_4 \cdot H_2O$ per 1250 ml. The liver mixture was distributed into eight 150 ml. flasks each containing 4 ml. of 0.188 M pyruvate in the side arms. 0.7 ml. of 0.094 M malonate was added to the liver suspension just before the air in the flasks was replaced by a mixture of 5 per cent carbon dioxide and 95 per cent oxygen. The pyruvate was then tipped in from the side cup, and the mixture was shaken in a water bath for 40 minutes at 40°.

The products (acetoacetate, α-ketoglutarate, citrate, malate, fumarate, and lactate (4, 16)) were fractionated as follows:

The combined reaction mixture (240 ml.) was cooled in an ice bath, and 50 ml. of ice-cold 2.0 per cent sodium bisulfite were

added to fix the keto acids. After 5 minutes the whole was deproteinized with 80 ml. of 15 per cent metaphosphoric acid. The bisulfite was added first and then the metaphosphoric acid, since an initial neutral pH is favorable for the formation of the bisulfite complex (3). To each 100 ml. of centrifuged solution, 60 gm. of $MgSO_4 \cdot 7H_2O$ were added, and the mixture was extracted continuously for 3 days with ether. A small amount of fresh bisulfite was added each day to replace loss of sulfur dioxide. The ether extract contained citric, malic, fumaric, succinic, and lactic acids; the bisulfite solutions contained the keto acids. The bisulfite solution was freed from sulfite by boiling the acid solution; then the keto acids were recovered by continuous extraction with ether for 24 hours. The α-ketoglutarate was separated from other keto acids by precipitation as the silver salt,[2] and the free acid was again obtained by ether extraction of a solution of the salt in dilute sulfuric acid. The α-ketoglutaric acid was oxidized with permanganate to carbon dioxide and succinic acid. The succinic acid was isolated as the silver salt after ether extraction. This acid and the carbon dioxide were analyzed for C^{13}. The carbon dioxide originates from the carboxyl group next to the carbonyl group, and the succinic acid from the remaining 4-carbon residue.

A partial separation of the citric, malic, fumaric, succinic, and lactic acids was accomplished by precipitation of the first four acids as the silver salts. The soluble silver lactate remains in the filtrate. The filtrate was treated with dilute hydrochloric acid to remove the silver ions and the solution was oxidized with permanganate (9, 29). The carboxyl carbon atom of lactate is converted to carbon dioxide and the α- and β-carbon atoms to acetaldehyde in this oxidation. These fractions were then analyzed for C^{13}.

The mixture of silver salts of citrate, malate, fumarate, and succinate was oxidized with permanganate. Succinate is stable,

[2] Silver salts were precipitated by addition of 5 ml. of 10 per cent silver nitrate to 25 ml. of the dicarboxylic acid solution and adjustment of the pH with NH_4OH (0.5 N) to a purple color to bromocresol purple. If the dicarboxylic acid solution is neutralized with NaOH solution and then the $AgNO_3$ solution is added, the precipitation is not quantitative, particularly of malate.

whereas the citrate, malate, and fumarate are broken down with conversion of the carboxyl groups to carbon dioxide. This carbon dioxide was analyzed for C^{13} to determine the fixed carbon in the carboxyl groups of these acids. In addition, the aldehyde formed during the oxidation was collected in bisulfite and analyzed for C^{13}. The α- and β-carbon atoms of malate are converted to acetaldehyde, whereas fumarate and citrate yield little or no acetaldehyde in this oxidation. In the case of citrate and fumarate, no attempt was made to determine the C^{13} in positions other than in carboxyl groups.

The succinic acid was recovered from the residue of the above oxidation by extraction with ether and precipitation as the silver salt. The entire molecule was oxidized to CO_2 and C^{13} determined.

No attempt was made to determine accurately the yields of products. Judging from the titration of the free acids, weight of the silver salts, and determination of citric acid by the method of Pucher *et al.* (18), the amounts when calculated on the basis of the whole dissimilation were as follows: α-ketoglutarate 0.57, succinate 0.42, fumarate and malate 0.77, citrate 0.046, and lactate 1.72 mM.

If one assumes (*cf.* "Discussion") that 2 molecules of pyruvate are used in the formation of each molecule of α-ketoglutarate, succinate, and citrate and one each for the fumarate, malate, and lactate, then approximately 80 per cent of the 5.6 mM of pyruvate utilized is accounted for. Evans (4) and others have not reported the formation of lactate in aerobic experiments in which malonate was an inhibitor. It seems probable that in our experiments there may have been accompanying dissimilation of pyruvate with formation of acetic acid which was not determined in these experiments.

The results of the mass spectrometric determination of the C^{13} in the respective isolated fractions are shown in Table I. The significance of these results will be considered later together with those of the following anaerobic experiment.

Fresh pigeon liver (25 gm.) was ground into 175 ml. of ice-cold salt solution and 20 ml. of distilled water in a 500 ml. Erlenmeyer flask. Anaerobiosis was assured by use of nitrogen gas. The mixture was warmed in a water bath to 40° and then 15 ml. of 0.35 M $NaHCO_3$ containing 9.61 per cent C^{13} and 15 ml. of

0.35 M pyruvate were added to give a final concentration of approximately 0.02 M pyruvate. The flask was attached through a mercury trap to a bead tower containing 10 ml. of 3 N alkali, and was shaken for 60 minutes at 40°. 100 ml. of 10 per cent metaphosphoric acid were then added, and the liberated carbon dioxide was removed by carbon dioxide-free nitrogen and collected in the alkali.

After centrifugation 285 ml. of solution were obtained from the 365 ml. of deproteinized suspension. 5 ml. were used to determine the residual pyruvic acid; the remaining 280 ml. portion was fractionated as follows:

The malic acid was separated from other acids by utilizing its slow extraction by ether. Succinic, fumaric, lactic, α-ketoglutaric, and pyruvic acids are completely extracted in 20 hours, whereas only 60 per cent of the malic acid is removed. Citric acid was not formed in significant amounts in this fermentation. The malic acid (40 per cent) remaining in the residual solution after 20 hours extraction was recovered by extraction with a second portion of ether for 4 days. The 20 hour ether extract was taken up in water and extracted again with ether for 20 hours. Approximately 24 per cent of the original malic acid remained in the residual water solution and 36 per cent was now in the extract. The residual solution was combined with the 4 day extract, and the 20 hour extract was taken up in water again and reextracted for 20 hours. This process was repeated five times and at the conclusion only 8 per cent of the malic acid remained in the extract.

The separated malic acid was further purified by precipitation as the silver salt. The free acid was recovered from the salt by ether extraction and was oxidized by permanganate to carbon dioxide and acetaldehyde for determination of the C^{13} in the carboxyl group and α- and β-carbon atoms respectively. That the acid oxidized was in reality largely malic acid is indicated by the recovery of approximately 2 molecules of carbon dioxide (0.14 mM) for each molecule of acetaldehyde (0.06 mM). The recovery of acetaldehyde from known malic acid was likewise about 80 per cent of the theoretical.

The succinic, fumaric, and lactic acids were separated from the keto acids by extraction from sulfite solution. Apparently

there was little or no α-ketoglutaric acid formed in the fermentation, for no insoluble silver salt was obtained from the keto acid fraction. Succinic and fumaric acids were separated from the lactic acid by precipitation as the silver salt. The carboxyl carbon atom and α- and β-carbon atoms of the lactate were obtained by oxidation of the filtrate with permanganate. An approximate equivalent yield of aldehyde (0.96 mM) and carbon dioxide (1.12 mM) was obtained; this indicates there was no significant contamination with oxidation products from other compounds.

The mixture of fumaric and succinic acids, obtained from the silver salt by extraction, was oxidized with permanganate. Carbon dioxide formed in the oxidation is considered to be carboxyl carbon from fumaric acid. Substantially no acetaldehyde was formed during the oxidation, which proves that the separation from malic acid was practically quantitative.

Succinic acid in the residue of the above permanganate oxidation was extracted with ether and then converted to a mixture of fumaric and malic acids by a heart muscle preparation. The mixture was then oxidized with permanganate. The resulting carbon dioxide and acetaldehyde correspond to the carboxyl and α- and β-carbon atoms of the succinic acid (*cf.* Wood *et al.* (29) for details of the methods). Calculation from the oxygen uptake by the heart muscle preparation showed that 0.19 mM of succinic acid was present; 0.36 mM of CO_2 and 0.11 mM of aldehyde were obtained in the permanganate oxidation. Since the proportions of malate and fumarate in the presence of fumarase are approximately 3:1, the yields of carbon dioxide and aldehyde are about theoretical.

Residual pyruvic acid was determined by the method of Straub (23). 53 per cent of the original pyruvate was fermented, and in the aliquot used for fractionation, 2.18 mM. Undoubtedly, most of the pyruvate unaccounted for was converted to acetic acid.

DISCUSSION

The data presented in Table I show that there are at least four primary facts that must be accounted for in the dissimilation of pyruvate by pigeon liver.

The fixation of carbon dioxide exclusively in the carboxyl groups of the 4-carbon dicarboxylic acids. This is clearly shown in Table I, for the α- and β-carbons of the 4-carbon dicarboxylic acids contained, within experimental error, the normal per cent of C^{13} (1.09 per cent), whereas the C^{13} content of the carboxyl carbons varied from 2.11 to 1.78 per cent. The latter figures indicate there was a substantial fixation of the $C^{13}O_2$ in the carboxyl position.

Table I

Distribution of Fixed Carbon in Products of Dissimilation of Pyruvate by Pigeon Liver

See the text for experimental conditions and methods of separation and degradation of the products.

Aerobic, malonate inhibitor	C^{13}	Anaerobic	C^{13}
	per cent		per cent
α-Ketoglutarate		Succinate	
Carboxyl adjacent to carbonyl group	2.17	Carboxyl carbons	2.11
Remaining 4 carbons of molecule	1.12	α- and β-carbons	1.11
Succinate	1.17	Malate	
Citrate, malate, and fumarate		Carboxyl carbons	1.78
Carboxyl carbons	1.90	α- and β-carbons	1.08
α- and β-carbons of malate	1.10	Fumarate	
Lactate		Carboxyl carbons	2.10
Carboxyl carbon	1.84	Lactate	
α- and β-carbons	1.12	Carboxyl carbon	1.74
		α- and β-carbons	1.09

$$\text{Per cent } C^{13} = \frac{\text{moles } C^{13}}{\text{moles } C^{12} + \text{moles } C^{13}} \times 100.$$

The formation of α-ketoglutarate containing fixed carbon in only one of its two carboxyl groups. The carboxyl carbon adjacent to the carbonyl group contained 2.17 per cent C^{13} and the remaining 4 carbons of the molecule a normal concentration of C^{13}.

The aerobic formation in the presence of malonate of succinate containing little or no fixed carbon, accompanied by formation of other 4-carbon dicarboxylic acids containing fixed carbon. The succinate in the aerobic experiment of Table I contained 1.17 per cent C^{13}; in other similar experiments we have observed

succinate formation in which the C^{13} content was actually normal and therefore contained no fixed carbon.

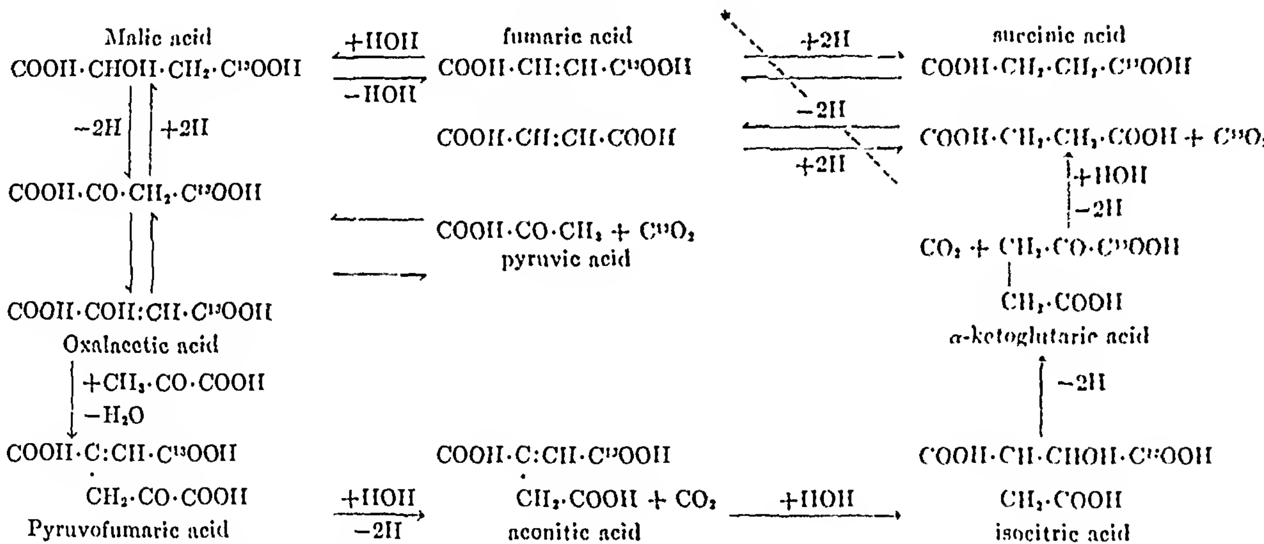
The aerobic and anaerobic formation of lactate containing fixed carbon in the carboxyl carbon and none in the α- and β-carbons. The carboxyl carbon contained 1.84 and 1.74 per cent C^{13} and the α- and β-carbons 1.12 and 1.09 per cent.

The accompanying scheme is supported by these facts. Formation of heavy carbon lactate is not shown in the scheme but is considered in the text.

With regard to the first point, the fixation reaction (Equation 1) is the only conversion thus far proposed in the literature that accounts for the formation from pyruvate by a malonate-insensitive reaction of 4-carbon dicarboxylic acids containing carbon of carbon dioxide exclusively in the carboxyl group. The condensation reactions involving either 2 molecules of pyruvate (24), or 1 of pyruvate and 1 of acetate (12), or 2 molecules of acetate (25), cannot account for the observations, even if one assumes an exchange of carbon dioxide with the carboxyl carbons to account for the presence of C^{13}. This is apparent, for these reactions pass through succinate prior to malate and fumarate formation. It is evident that the malate and fumarate formed in the aerobic experiment of Table I did not pass through succinate, for the equilibrium between fumarate and succinate was blocked effectively, as shown by the marked difference in the C^{13} concentration of the respective compounds.

Prior to the present investigation the evidence for fixation of carbon dioxide by animal tissue according to Equation 1 was largely based on investigations with bacteria (27, 29). The statement of Krebs (14), "This [Evans and Slotin's demonstration of fixed carbon in α-ketoglutarate] completes the proof of the occurrence of reaction 9 [$COOH \cdot CO \cdot CH_3 + CO_2 = COOH \cdot CO \cdot CH_2COOH$] in pigeon liver," must be accepted with considerable reservation, particularly since the position of the fixed carbon in α-ketoglutarate was not known at this time. The evidence presented in this paper more nearly completes the proof, since the fixed carbon has been located directly in the 4-carbon dicarboxylic acids and it is exclusively in the carboxyl group. It is to be emphasized, however, that even in the case of bacteria, Equation 1 is only a preliminary representation of 3-

Dissimilation of Pyruvate by Pigeon Liver



* Reaction is inhibited by malonate.

and 1-carbon addition. It is probable that phosphorylated intermediates are involved and the reaction is more complex than pictured. Recent results by Krampitz *et al.*[1] obtained with bacteria provide direct evidence, however, that oxalacetate may be a component of the reaction. Although unable to accomplish a synthesis of oxalacetate from pyruvate and carbon dioxide, they demonstrated an exchange of $C^{13}O_2$ with the carboxyl adjacent to the methylene carbon of oxalacetate by use of a newly discovered enzyme, which decarboxylates this compound to pyruvic acid and carbon dioxide. This exchange reaction is essentially 3- and 1-carbon addition. These authors on the contrary could not demonstrate an exchange during enzymic decarboxylation of pyruvate or α-ketoglutarate.

Of interest is the fact that Krebs and Eggleston (16) have presented evidence which they believe demonstrates the necessity of cocarboxylase for catalysis of the fixation reaction. Results of Krampitz and Werkman (11) may not support this idea. They found with the bacterial preparation that Mg^{++} is essential but not cocarboxylase for decarboxylation of oxalacetate to pyruvate.

Relative to the second point, *i.e.* that the fixed carbon is in only one carboxyl of α-ketoglutarate, it should be pointed out that, although the accompanying scheme is adaptable to this fact, the evidence for the suggested reactions is largely drawn from analogy with Krebs' results on pyruvate oxidation by pigeon breast muscle. It is fairly certain that α-ketoglutarate is formed by combination of a 4-carbon dicarboxylic acid and pyruvate in this dissimilation (15). The exact mechanism of the reactions involved in this conversion is not known, however. This fact is emphasized by Krebs' erroneous prediction (14) that fixed carbon would be found in both carboxyls of α-ketoglutarate in the case of pigeon liver dissimilation. Actually the fixed carbon is confined to one carboxyl (30, 5) (Table I). This fact definitely precludes citrate as an intermediate, since α-ketoglutarate derived from the symmetrical citrate molecule should contain equal amounts of fixed carbon in its two carboxyl groups. The scheme probably represents roughly the carbon structure of the compounds involved in α-ketoglutarate synthesis, but not

the actual intermediates. It is quite likely that phosphorylation reactions play an important part in these conversions and that aconitic, isocitric, etc., acids do not occur as intermediates but phosphorylated derivatives. The fact holds, however, that assuming a synthesis of 4-carbon dicarboxylic acids by 3- and 1-carbon addition and formation of α-ketoglutarate by union of this acid with pyruvate, the occurrence of fixed carbon in one carboxyl of α-ketoglutarate can be accounted for. No scheme other than the Krebs cycle has been proposed for α-ketoglutarate synthesis which accounts for this location of fixed carbon.

The third point, that succinate formed aerobically in the presence of malonate contains little or no fixed carbon, is of considerable importance. In fact Krebs (13) refers to the oxidative formation of succinate from oxalacetate in the presence of malonate as the crucial experiment. The experiment is crucial, for it shows that there is an *oxidative* reaction leading from oxalacetate to succinate, provided one can assume that anaerobic formation of succinate by reduction of oxalacetate is inhibited effectively. Critics of the Krebs cycle (1, 13) contend this assumption is without adequate proof. The results in Table I show that in the case of pigeon liver the inhibition by malonate was effective and the succinate did not arise by anaerobic reduction over malate and fumarate; for if such had been the case the C^{13} concentration would be approximately the same in each compound as it was in the anaerobic experiment in which malonate was not added. It is clear therefore that there are two mechanisms for the formation of 4-carbon dicarboxylic acids. The one is quite probably by 3- and 1-carbon addition, is not inhibited by malonate, and the acids contain fixed carbon. The other is by an oxidative process and the succinic acid does not contain fixed carbon. This then removes one of the major criticisms of the proof of the Krebs cycle, at least as applied to liver dissimilation. Certain details of the Krebs cycle may be wrong, but there is good basis for assuming that the general framework is correct.

The fourth point, fixation of carbon dioxide in the carboxyl of lactate, requires further study before its significance can be evaluated fully. It is probable that the carbon dioxide is fixed initially by 3- and 1-carbon addition and that pyruvic acid con-

taining heavy carbon is formed by the following dynamic equilibria involving a shift of the hydroxyl of enol oxalacetic acid and a subsequent decarboxylation.

$$(2)\quad C^{13}O_2 + CH_3\cdot CO\cdot COOH = C^{13}OOH\cdot CH{:}COH\cdot COOH = \\ C^{13}OOH\cdot COH{:}CH\cdot COOH = C^{13}OOH\cdot CO\cdot CH_3 + CO_2$$

The pyruvic acid thus formed is reduced to lactic acid. It is interesting in this connection that Slade *et al.* (21) have found that CO_2 is fixed in lactic acid formed by most of the bacteria tested. A few bacteria did not fix carbon in lactate, however. It is significant that in one fermentation the succinate contained fixed carbon but not the lactate. This fact may indicate the carbon is not fixed in lactate by the above equilibria (Equation 2), for if such was the case it would be expected that, whenever 4-carbon dicarboxylic acids were formed containing heavy carbon, heavy carbon pyruvate and lactate would likewise occur. The possibility is left open that the heavy carbon lactate is not formed by the above equilibria but is perhaps formed by 2- and 1-carbon addition, though at present all evidence is to the contrary.

Solomon *et al.* (22) have found in the synthesis of glycogen from lactate that carbon dioxide is fixed in glycogen. The reverse of this reaction, glycolysis, may also involve fixation of carbon dioxide in lactate. Numerous investigators have indicated their belief in the occurrence of other mechanisms of glycolysis than that proposed by Meyerhof. Perhaps one mechanism may involve fixation of carbon dioxide, whereas the other is independent of fixation.

The accompanying scheme indicates that carbon dioxide fixation is an essential part of the mechanism, and in the presence of malonate all fumarate and malate arise directly by fixation. Therefore, one (or perhaps both, if Equation 2 occurs) of the carboxyl groups of fumarate and of malate and also the carboxyl adjacent to the carbonyl in α-ketoglutarate should contain entirely fixed carbon. Theoretically it would be expected, then, that the C^{13} content of the $NaHCO_3$ plus gaseous carbon dioxide would be approximately equivalent to that in the particular carboxyl carbon atoms under consideration. The C^{13} in the sodium bicarbonate plus gaseous CO_2 was not determined at the con-

clusion of the aerobic dissimilation but the calculated value is 4.91.[3] A number of factors may contribute to this discrepancy between the C^{13} content of the carboxyl groups and that of the $C^{13}O_2$. First, the CO_2 formed within the cell from the substrate may not come to equilibrium with the $C^{13}O_2$ outside of the cell, and therefore the CO_2 available to the cell for fixation has a lower content of C^{13}. It is evident that the relative concentration of C^{13} within the cell and in the medium outside of the cell will be dependent largely on the permeability of the cell membrane and the rate of formation of $C^{12}O_2$ from the substrate. Another factor is that there is a small amount of each compound such as α-ketoglutarate, succinate, etc., present in the tissue prior to the exposure to $C^{13}O_2$. Since in the method of fractionation, these compounds are included with those formed subsequently by the fixation reaction, there is, consequently, some dilution of the C^{13}. A third factor is suggested by results from the anaerobic fermentation in which the C^{13} content of the final sodium bicarbonate plus gaseous CO_2 was determined. The observed value was 6.02 per cent C^{13}. Actually 3.82 mM of CO_2 with a normal C^{13} content would be required to produce such a dilution of the CO_2 of the original $NaHCO_3$ which contains 9.61 per cent C^{13}. Determination showed that only 1.42 mM were produced. This apparently indicates that the $C^{13}O_2$ was diluted by exchange with carbon from the pigeon liver itself. It would be interesting to know more about the exchange reactions occurring in the liver tissue. Evans and Slotin (7) and Rittenberg and Waelsch (19) have shown that the carbon of urea is derived from carbon dioxide; perhaps there is an exchange of C^{13} and C^{12} by this reaction in our experiments. The exchange may also occur in the carboxyl group of amino acids, particularly with the 4-carbon dicarboxylic acids through the combined action of transaminase (2) and the enzyme which

[3] The final concentration of C^{13} in the bicarbonate and gaseous CO_2 has been calculated on the basis that the original sodium bicarbonate was diluted by the following amounts of $C^{12}O_2$: 2.7 mM from the 5 per cent CO_2 and 95 per cent oxygen mixture, 3.75 mM produced by fermentation of the pyruvate. It is assumed that 3 molecules of CO_2 were formed with each molecule of succinate, 2 with each molecule of α-ketoglutarate, and 1 with each of citrate and acetate. Acetate production is considered equal to the lactate.

catalyzes exchange in oxalacetate. At any rate because of the uncertainties as to the C^{13} content of the CO_2 available to the cell for fixation, it is not possible to predict the concentration of C^{13} to expect in the carboxyl groups. Furthermore, it is evident that even though the C^{13} content of the required carboxyl carbons and the gaseous and sodium bicarbonate carbon were equivalent, this would not unequivocally prove that carbon fixation was an essential reaction. The initial reaction in oxalacetate synthesis could occur, for example, by some other reaction than fixation and following this the carboxyl groups could come to equilibrium with $C^{13}O_2$ by the enzymic exchange reaction of Krampitz *et al.*[1] The C^{13} concentration would be the same by either pathway. There is ample evidence, however, that carbon dioxide does not function solely in non-essential exchange reactions, for it is known, for example, to be necessary for the growth of microorganisms (20) and in the reduction of methylene blue by dehydrogenases (10). Obviously due precaution must be taken in deciding the course of a reaction from the location of fixed carbon in the molecule. Our position is that 4-carbon dicarboxylic acids can be formed by fixation, but nothing is known at present as to the necessity of this reaction in the dissimilation of pyruvate by liver.

SUMMARY

The dissimilation of pyruvate by pigeon liver occurs with accompanying fixation of carbon dioxide. By use of $C^{13}O_2$ the fixed carbon has been shown to be exclusively in the carboxyl groups of the 4-carbon dicarboxylic acids (malate, fumarate, and succinate), the carboxyl adjacent to the carbonyl of α-ketoglutarate, and the carboxyl of lactate. Aerobically in the presence of malonate succinate is formed which contains little or no fixed carbon. It is proposed that the 4-carbon dicarboxylic acids are formed by two mechanisms, one reductive through the carbon fixation reaction, the other oxidative by a tentative and modified Krebs cycle which does not involve citric acid. The scheme accounts for the observed positions of the fixed carbon and the aerobic formation in the presence of malonate of succinate not containing fixed carbon. Possible mechanisms of fixation of carbon in lactate are considered.

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THE FORMATION OF ACETONE BODIES FROM ACETIC ACID*

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There are many compounds which in the course of biological oxidation are regarded as capable of forming acetic acid and, therefore, the metabolic behavior of this substance is of considerable interest. When acetic acid is fed to animals, it is readily utilized (1) and the question arises as to whether this utilization is by direct oxidation or through the formation of intermediate compounds which may in turn be oxidized. One type of intermediary conversion that has been studied is the formation of acetoacetic and β-hydroxybutyric acids from acetic acid. Investigations on perfused livers (2), liver slices (3), and intact animals (4) have all shown that acetic acid causes an increased production of the acetone bodies. However, evidence gained in this manner leaves some doubt as to whether the acetone bodies were formed directly from acetic acid or whether the administration of acetic acid stimulated acetone body formation from some other precursor. In order to determine which one of these two mechanisms is effective in the formation of acetone bodies, the following experiments, with the heavy isotope of carbon, C^{13}, were performed.

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† The experimental data are taken from a thesis submitted to the Faculty of the Graduate School of the University of Minnesota by Marian Swendseid in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

EXPERIMENTAL

Acetic acid containing excess heavy carbon in the carboxyl carbon atom was synthesized by the Grignard method from CH_3MgI and CO_2 containing excess C^{13}. A 1 M concentration of the heavy acetic acid containing 4.3 per cent C^{13} in a 0.9 per cent sodium chloride solution was fed by stomach tube to two female rats weighing approximately 100 gm. Two comparable rats of the same body size were given 0.9 per cent sodium chloride solution alone. The rats, which had been previously fasted for 24 hours, were given 1 cc. of the test solutions per sq. dm. of body surface twice daily for 3 days. Individual 24 hour urine specimens were collected and the samples were treated with copper and calcium hydroxide according to the method of Van Slyke (5). It was found that the amount of acetone bodies excreted by the rats receiving sodium chloride alone was too small for C^{13} analysis; so the urines from the two controls were combined. One-half of the copper lime filtrate was boiled with mercuric sulfate reagent and potassium dichromate as described by Van Slyke (5) for the determination of urine acetone bodies. The Denigès mercury-acetone precipitate resulting from this treatment was weighed for the calculation of daily acetone body excretion. It was then oxidized to CO_2 with persulfate (6) and the CO_2 collected for C^{13} analysis by the mass spectrometer. The remaining portion of the copper lime filtrate was acidified and the acetone bodies extracted with butyl alcohol by the method of Morehouse (7). This extract which contains acetoacetic acid, β-hydroxybutyric acid, and some other contaminating organic acids was oxidized with persulfate (6) and the evolved CO_2 analyzed for C^{13}.

A similar experiment was performed in which fasting rats were given sodium bicarbonate containing the heavy isotope of carbon. As in the acetic acid experiment, both the Denigès mercury-acetone precipitate formed in the regular Van Slyke acetone body determination and the butyl alcohol extracts of the copper lime urine filtrate were oxidized with persulfate and the CO_2 analyzed for C^{13}. In this experiment 1 cc. of either 1 M heavy sodium bicarbonate containing 4.5 per cent C^{13} or 0.9 per cent sodium chloride per sq. dm. of body surface was administered twice daily to fasting rats for 3 days.

All of the values for the C^{13} content of the acetone body prepa-

rations reported in Tables I and II are related to a value of 1.09 per cent C^{13} of a standard solution of Merck's reagent sodium bicarbonate. Nier and coworkers (9, 10) have shown that variations occur in the relative abundance of the carbon isotopes obtained from natural sources. It has been found in this laboratory (unpublished results) that the natural abundance of C^{13} from animal tissues averages lower than that obtained from mineral

TABLE I

Heavy Carbon Content of Urinary Acetone Bodies from Fasting Rats Fed Heavy Acetic Acid

Day of fasting	Rat No.	Body surface average	Treatment*	Acetone body excretion per 100 sq. cm. body surface per day (calculated as acetone)	C^{13} in Denigès ppt.†	C^{13} in butyl alcohol extract†
		sq. dm.		*mg.*	*per cent*	*per cent*
1st	1, 2	2.4	NaCl	1.6	1.07	1.07
	3, 4	2.5	AcOH	4.9	1.12	1.14
2nd	1, 2		NaCl	1.6		1.08
	3		AcOH	18.8	1.13	1.27
	4		"	12.7	1.13	1.20
3rd	1, 2		NaCl	3.4	1.08	1.09
	3		AcOH	19.6	1.17	1.37
	4		"	2.9	1.08	1.10

Body surface was calculated from the formula of Carman and Mitchell (8).

* Each rat was fed either 0.15 M sodium chloride or 1 M acetic acid in doses of 1 cc. per sq. dm. of body surface twice daily.

† $C^{13} = \dfrac{\text{moles } C^{13}}{\text{moles } C^{12} + \text{moles } C^{13}} \times 100.$

sources and has a value relative to the standard sodium bicarbonate solution mentioned above of 1.06 ± 0.04 per cent C^{13}.

The results of the heavy acetic acid experiment are shown in Table I. It will be seen that with one exception (Rat 4 on the 3rd day of fasting) the administration of acetic acid resulted in an increased excretion of acetone bodies. Furthermore, accompanying this increased excretion there was an excess of C^{13} in both the Denigès precipitate and the butyl alcohol extract over that found in the controls given sodium chloride. In the one case in which acetone body excretion did not increase after acetic acid

there was no increase in C^{13} in the two acetone body preparations. A severe diarrhea developed in this rat on the 3rd day and it is possible that intestinal absorption was impaired.

While these results leave no doubt as to the transfer of carboxyl carbon of acetic acid to acetoacetic and β-hydroxybutyric acids, it is possible that the acetic acid carboxyl was converted to CO_2 and that carbon in this form was incorporated in the acetone bodies. In order to test this possibility a second group of two rats was given sodium bicarbonate containing excess C^{13} and the

TABLE II

Heavy Carbon Content of Urinary Acetone Bodies from Fasting Rats Fed Heavy Sodium Bicarbonate

Day of fasting	Rat No.	Body surface average	Treatment*	Acetone body excretion per 100 sq. cm. body surface per day (calculated as acetone)	C^{13} in Denigès ppt.†	C^{13} in butyl alcohol extract†
		sq. dm.		*mg.*	*per cent*	*per cent*
1st	1, 2	2.4	NaCl	1.1	1.08	1.07
	3	2.4	$NaHCO_3$	2.5	1.09	1.08
	4	2.5	"	6.2	1.07	1.07
2nd	1, 2		NaCl	1.5	1.07	1.06
	3		$NaHCO_3$	2.7	1.07	1.07
	4		"	10.0	1.08	1.08
3rd	1, 2		NaCl	2.4	1.07	1.09
	3		$NaHCO_3$	2.9	1.08	1.08
	4		"	14.4	1.07	1.08

*Each rat was fed either 0.15 M sodium chloride or 1 M sodium bicarbonate in doses of 1 cc. per sq. dm. of body surface twice daily.

†See Table I.

excretion and distribution of C^{13} in the acetone bodies were again followed. Sodium chloride was given the two controls. The results are given in Table II. Both rats given sodium bicarbonate showed an increased ketonuria over the controls, Rat 4 exhibiting the greatest excretion. Neither the Denigès precipitate nor the butyl alcohol extract of the urines of the heavy sodium bicarbonate-fed rats showed an excess C^{13}. Therefore the excess C^{13} found in the urinary acetone body preparations of the acetic acid-fed rats must have come from a direct transfer of the carboxyl.

carbon rather than through the intermediate formation of CO_2. It has long been recognized that sodium bicarbonate administration causes an increased ketosis. Recently MacKay *et al.* (11) have presented evidence that this ketogenic activity of alkali is brought about by a secondary disturbance in the carbohydrate metabolism. The results presented here lend support to this conclusion in that it is shown that the carbon of sodium bicarbonate does not take part in acetone body synthesis.

In the Van Slyke procedure for acetone body determination, acetoacetic and β-hydroxybutyric acids are oxidized to acetone and the formed acetone is combined with mercury to form an insoluble addition compound. Thus the acetone does not include the carboxyl carbon of either β-hydroxybutyric or acetoacetic acid. On the other hand, the butyl alcohol extract should contain the entire molecule of these two acids. It would be expected that the latter preparation would contain more C^{13} than the former if the acetone bodies are formed by the condensation of 2 moles of acetic acid. It will be seen in Table I that this is the case. However, the C^{13} content of the butyl alcohol extract is much higher than would be expected from the stoichiometric relationship. While no definite explanation can at present be given, it should be remembered that the butyl alcohol extract as prepared probably contains organic acids other than acetoacetic or β-hydroxybutyric and that acetic acid might be taking part in the synthesis of these other compounds.

SUMMARY

By the use of the heavy isotope of carbon, C^{13}, it has been shown that acetic acid takes part in the synthesis of the acetone bodies in the fasting rat. In a similar manner it has been shown that the carbon of sodium bicarbonate does not enter the acetone bodies formed by the fasting rat.

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A COMPARISON OF THE ACETONE BODY METABOLISM OF THE LACTATING MAMMARY GLAND OF THE NORMAL COW WITH THAT OF THE COW WITH KETOSIS

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The mammary gland of the cow is unique as an organ for purposes of experimental study of various metabolic processes. Protein, carbohydrate, and fat synthesis all take place at a very rapid rate in an organ almost isolated from the remainder of the body except for the circulatory and nervous systems and a number of supporting tissues. Because of the accessibility of the blood vessels, both arterial and mammary venous blood can be drawn without recourse to surgery, and, with proper technique, without excitation to the animal. One of the most important steps in the development of the arteriovenous method of study of milk secretion has been the observations of the effect of excitation upon blood concentration changes in the gland and the bearing of such changes upon the validity of results obtained in arteriovenous studies (Shaw and Petersen (1)). It was demonstrated that the concentration of hemoglobin in the passage of the blood through the gland rarely exceeded 1 per cent in the undisturbed animal and in most cases the changes were much less. With noticeable disturbance to the animal there were significant changes in blood concentration and coincidentally the arteriovenous differences for fat, protein, oxygen, and carbon dioxide showed values which were obviously untenable. Blood glucose, amino acids, and acetone bodies are not affected so seriously. Recent data (unpublished) demonstrate that arteriovenous differences of calcium and phosphorus are affected very materially by conditions which result in even slight changes in the concentration of the blood traversing the gland. As a result of

this work we are in a much better position to evaluate the results obtained from arteriovenous studies. The experiments in which no detectable changes in blood concentration occur give the most consistent results and are considered to represent the true picture in the completely undisturbed animal. The method, while accurate to only 0.5 per cent on the absolute basis, can be used to detect much smaller differences between samples. Since the concentration changes probably are due to changes in blood pressure, the observations may be expected to apply to similar studies upon other organs of the body.

In a comprehensive study of the quantity of glucose and lactic acid and of the quantity of blood fat utilized by the lactating mammary gland (Shaw and Petersen (2, 3)) we were led to conclude that the gland must obtain much of its energy from the burning of fat. On the basis of the quantity of blood calcium taken up by the active gland per 100 cc. of blood, and the per cent of calcium in the milk, and the quantity of blood glucose and lactic acid taken up by the active gland per 100 cc. of blood, and the per cent of lactose in the milk, our calculations showed that practically all of the glucose and lactic acid are needed for the synthesis of lactose, leaving very little for energy purposes or for the synthesis of fat. Similar calculations on a large number of experiments indicated that sufficient blood fat was taken up by the mammary gland to account for all of the milk fat. As a result we postulated that some of the longer chain fatty acids were oxidized for energy purposes and that the short chain fatty acids peculiar to milk fat were formed by oxidation and subsequent reduction of the longer chain fatty acids, possibly in the manner proposed by Hilditch *et al.* (4, 5). Graham *et al.* (6) on the other hand concluded that the energy for milk secretion was derived from carbohydrate.

What is believed to be direct evidence of the oxidation of fat by the mammary gland was reported by Shaw and Knodt (7). In a series of arteriovenous studies it was shown that a relatively large quantity of β-hydroxybutyric acid is used by the lactating gland. Also it was observed that the fraction consisting of acetoacetic acid and acetone is not used by the active gland. Two alternative possibilities were proposed as to the rôle of β-hydroxybutyric acid in milk secretion, (1) that it is used as a source of energy, and (2) that it is the precursor of the short chain fatty acids of milk. Later

evidence was presented favoring the latter view (8). More recently we have found that the short chain fatty acids of milk fat decrease very markedly in ketosis in dairy cows (9). Since the β-hydroxybutyric acid of the blood is considerably above normal in ketosis, this raises a question as to the validity of such a suggestion and favors our alternative suggestion that it is oxidized for energy purposes.

In a further study of the fat metabolism of the mammary gland, dairy cows with ketosis were selected as experimental subjects because of the relatively large quantity of acetone bodies in the blood. These data indicate that not only β-hydroxybutyric acid but also considerable quantities of other fat are oxidized by the lactating mammary gland of the cow for energy purposes.

EXPERIMENTAL

Arterial and mammary venous bloods were drawn from cows in a state of ketosis in the manner previously described (1). Blood acetone bodies were determined by the method of Barnes and Wick (10) with the same precautions in the handling of the blood samples as in the previous experiments (7). Oxygen and carbon dioxide were determined by the methods of Van Slyke and Neill (11) and hemoglobin by the method of Evelyn and Malloy (12). The experimental animals used were field cases of dairy cows with spontaneous ketosis. In eight arteriovenous studies on seven cows with ketosis both the acetone bodies and oxygen utilization of the lactating gland were determined. These data are presented in Table I. The acetone body utilization is calculated as β-hydroxybutyric acid, since the fraction consisting of acetoacetic acid and acetone is not used by the active gland, as shown in Table II and elsewhere (7). For purposes of comparison the utilization of β-hydroxybutyric acid and oxygen by the glands of normal cows also is presented in Table I. In fifteen experiments on normal cows in which there was no change in blood concentration in the mammary gland there was a mean utilization of 1.83 ± 0.246 mg. per cent of β-hydroxybutyric acid. In the experiments on the cows with ketosis there was a mean utilization of 4.11 ± 0.310 mg. per cent or over 100 per cent more than the utilization by the gland of the normal cow. Assuming that β-hydroxybutyric acid is oxidized by the gland for energy purposes, it would be expected

TABLE I

Comparison of Utilization of β-Hydroxybutyric Acid and Oxygen by Lactating Mammary Gland of Normal Cow with That of Cow with Ketosis

Cow	Blood concentration changes	Total acetone bodies (as acetone)		Difference (as β-hydroxybutyric acid)	Oxygen consumed	Assuming β-hydroxybutyrate burning		R.Q.
		Arterial	Venous			Oxygen required	Per cent of oxygen consumed needed	
Cows with ketosis								
	per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*vol. per cent*	*vol. per cent*		
Do.	0.59	20.10	18.16	4.06	3.53	3.89	110.19	1.38
Sp.	0.35	23.52	21.60	3.97	3.72	3.85	103.49	0.98
Wr.	0.47	34.10	31.91	4.52	4.23	4.38	103.54	1.15
Sh.	0.25	35.80	34.09	3.53	2.96	3.42	115.54	1.34
A.	0.00	29.15	27.22	3.99	4.82	3.87	80.29	0.82
"	0.87	37.63	35.07	5.29	3.84	5.13	133.59	1.20
Ne.	0.00	46.13	43.69	5.04	2.91	4.89	168.04	1.24
He.	0.00	11.14	9.93	2.50	3.09	2.43	78.64	1.09
Average	0.32	29.70	27.71	4.11	3.64	3.98	111.67	1.15
Standard error				±0.310	±0.235			±0.057
Normal cows								
Average		3.40	2.52	1.83*	4.83†	1.78	36.85	
Standard error				±0.246	±0.156			

* Fifteen cows.

† Unpublished data of Petersen and Shaw on fourteen cows.

TABLE II

Arteriovenous Differences of Acetoacetic Acid + Acetone of Lactating Gland of Cow with Ketosis

Cow	Acetoacetic acid + acetone (as acetone)			Blood concentration change
	Arterial	Venous	Difference	
	mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*
Ki.	12.38	12.67	+0.29	0.50
Ne.	14.14	14.32	+0.18	0.00
"	13.14	13.38	+0.24	0.00
He.	10.89	10.65	−0.24	0.00
Average	12.64	12.76	+0.12	

that with the increased uptake by the gland of the animal with ketosis there would be a corresponding increase in oxygen utilization, unless oxygen which normally is utilized for other purposes is released for the increased burning of β-hydroxybutyric acid. In fourteen experiments on normal cows in which changes in blood concentration in the gland did not exceed 0.5 per cent, there was a mean utilization of 4.83 ± 0.156 volumes per cent of oxygen. The mean utilization of oxygen by the glands of the cows with ketosis was 3.64 ± 0.235 volumes per cent, or actually less than normal.

Assuming that 0.97 cc. of oxygen is required for the complete combustion of 1.0 mg. of β-hydroxybutyric acid, an average of 3.98 cc. of oxygen per 100 cc. of blood would be required to oxidize completely the β-hydroxybutyric acid used by the glands in ketosis. This is greater than the total amount of oxygen available but probably is within experimental error. In the case of the glands of the normal cows only 36.85 per cent of the available oxygen would be needed to oxidize completely the β-hydroxybutyric acid utilized. We reported in an earlier communication (7) that 42 per cent of the available oxygen would be needed for the complete combustion of this material. The values given in Table I are based on an extension of the previous studies and involve a larger number of determinations in which no blood concentration changes occurred.

It is apparent then that in the gland of the normal cow approximately 63 per cent of the oxygen taken up by the gland is available for the oxidation of some substance or substances other than the β-hydroxybutyric acid derived directly from the blood. This could be explained by assuming that with the onset of ketosis and the resulting increase in blood acetone bodies there is a change from the oxidation of both β-hydroxybutyric acid and other fat to the sole oxidation of β-hydroxybutyric acid. This explanation would account for the smaller quantity of oxygen taken up by the gland in ketosis, since a shift from the oxidation of fatty acids to an oxidation of β-hydroxybutyric acid would require less oxygen. Unpublished data show that the utilization of glucose and lactic acid by the gland of the cow in ketosis is approximately normal even though both substances are below normal in the blood. It therefore appears that the increased utilization of β-hydroxybutyric acid by the gland of the cow with ketosis is not due to a decrease in the oxidation of carbohydrate but is due to a change from the oxi-

dation of other fat to the sole oxidation of β-hydroxybutyric acid. If this is the case, we must assume that the normal gland obtains much of its energy from the oxidation of fat in which approximately 37 per cent of its available oxygen is used for the oxidation of β-hydroxybutyric acid and the other 63 per cent for the oxidation of other fat.

If the mammary gland of the normal cow oxidizes β-hydroxybutyric acid for energy purposes, the increased utilization of β-hydroxybutyric acid for energy purposes in ketosis might be expected from the work of Barnes *et al.* (13–15), Dye and Chidsey (16), and Wick and Drury (17) in which it was shown that the rate of utilization is dependent on the concentration of this substance in the blood. The latter workers reported that the upper limit of utilization appeared to be reached when the oxidation of β-hydroxybutyric acid used approximately 90 per cent of the oxygen consumed by the animal. In the case of the mammary gland of the cow with ketosis it appears that the upper limit of utilization is not reached until practically all of the oxygen taken up by the gland has been used up. The lesser amount of oxygen used by the gland in ketosis and the increased uptake of β-hydroxybutyric acid indicate that the rate of utilization of this substance is dependent upon the energy requirements of the gland and that the rate of oxygen utilization is dependent upon the kind and quantity of substance available for oxidation. In the normal cow in which insufficient β-hydroxybutyric acid is available to provide all the needed energy the gland probably turns to the oxidation of other fat with the resulting increase in the oxygen utilization. That the blood flow and not the arteriovenous differences vary with a change in the level of milk production was first demonstrated by Shaw, Boyd, and Petersen (2) and indicates that any change in the activity of the secretory tissue of the gland during ketosis probably is coincidental with a corresponding change in the volume of blood traversing the gland and should not affect materially the arteriovenous differences of those substances used in direct synthesis. It therefore appears that the decreased oxygen uptake is not due to the decline in milk flow which often occurs in ketosis.

If fat other than β-hydroxybutyric acid is oxidized in the gland, it is quite possible that the short chain fatty acids of milk fat are derived from such oxidation, followed by reduction, as previously

suggested. The marked decrease in the short chain fatty acids in ketosis and in complete inanition could both be explained on this basis, for with the increase in acetone bodies which occurs in both cases there would be a decrease in the oxidation of other fat and a corresponding decrease in the formation of the short chain fatty acids of milk.

The metabolic processes of the gland which are responsible for the high respiratory quotient of the normal gland, as reported by Graham *et al.* (6), Shaw and Petersen (18), and Reineke *et al.* (19), do not appear to have been altered materially in ketosis, as shown by the mean value of 1.15 ± 0.057 in Table I. The oxidation of a considerable quantity of fat would indicate that some unknown metabolic processes may be taking place at a rapid rate to account for the high respiratory quotient. However, considering the difficulty involved in attempting to evaluate respiratory quotient values even in relatively simple systems, it is deemed advisable to withhold judgment as to the possible meaning of such values until considerably more is known about the various reactions taking place in the gland.

The simultaneous increase in β-hydroxybutyric acid utilization and decrease in the short chain fatty acids of milk clearly demonstrate that this substance is not the precursor of these fatty acids.

It is of interest to note that neither the gland of the normal nor of the cow in a state of ketosis used acetoacetic acid. It is apparent that in the case of the mammary gland, at least, the acetoacetic acid must be converted to β-hydroxybutyric acid elsewhere in the body before it is utilized, and suggests the interesting possibility that a similar conversion may have to take place for the oxidation of this substance by other tissues of the body.

SUMMARY

1. The lactating mammary gland of the cow in ketosis used over 100 per cent more β-hydroxybutyric acid per 100 cc. of blood traversing the gland than the gland of the normal cow. Simultaneously there appears to be a decrease in oxygen utilization.

2. Practically all of the oxygen taken up by the lactating gland of the cow with ketosis would be needed for the complete combustion of the β-hydroxybutyric acid removed from the blood by the gland.

3. In the normal gland only 37 per cent of the oxygen consumed would be required for the complete combustion of the β-hydroxybutyric acid used. It is suggested that much of the energy for milk production in the normal gland is derived from the oxidation of fat in which approximately 37 per cent of the oxygen consumed is used in the oxidation of β-hydroxybutyric acid, while the remaining 63 per cent is used in the oxidation of other fat.

4. Acetoacetic acid is not utilized by the active mammary gland of either the normal cow or the cow with ketosis.

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THE POLAROGRAPHIC ESTIMATION OF CYSTINE IN URINE

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Urine of healthy persons contains small concentrations of cystine and possibly other substances of low molecular weight, which contain —S—S— and —S—H groups. Colorimetric estimations of cystine in urine were reported by Shinohara and Padis (1), Medes (2), Kennedy *et al.* (3), and Sullivan and Hess (4). Brdička (5) describes catalytic steps and waves on polarographic current voltage curves, which are caused by organic mercapto and mercaptoamino acids and cystine- and cysteine-containing proteins in suitable cobalt buffer solutions. The present paper deals with the application of this method to the estimation of cystine[1] in urine, and a comparison with the Sullivan-Hess colorimetric method.

The polarographic effect of protein —S—S— and —S—H groups is observed in ammoniacal buffers containing di- or trivalent cobalt salts; whereas cystine and cysteine react only in the presence of divalent cobalt. The effect of cystine and cysteine may thus be eliminated by the use of trivalent cobalt salts and urinary proteins can be estimated polarographically in spite of the great excess of urinary cystine (6). The latter may be estimated with divalent cobalt in dilutions at which the polarographic effect of urinary proteins becomes negligible. Separate estimations of cystine and protein can therefore be carried out without previous chemical or physical separation.

[1] The term cystine will be used throughout this paper for the total of cystine plus cysteine, because by the polarographic method and the colorimetric method, involving the use of NaCN, there is no discrimination between the reduced and the oxidized form.

For the estimation of cystine 0.5 ml. of urine is added to 200 ml. of a solution 0.003 N in $CoCl_2$, 0.1 N in NH_4Cl, and 0.1 N in NH_3. A polarographic record of the solution exposed to the air is made at from -1.2 to -1.8 volts (galvanometer sensitivity 0.5 microampere per mm.). The drop time of the cathode should be about 3 seconds in distilled water. A 14 gage silver wire is conveniently used as anode.

The resulting polarographic wave showed the same shape and deposition potential as did pure cystine solutions, and the same

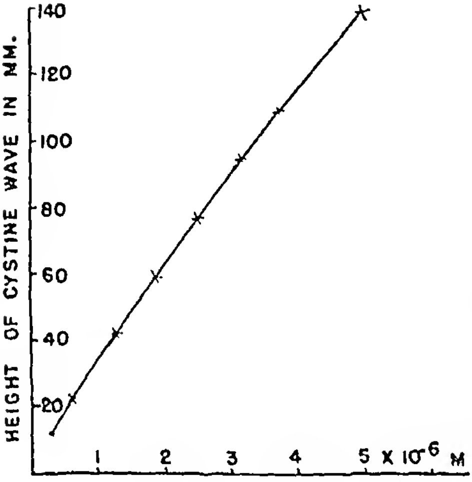

Fig. 1

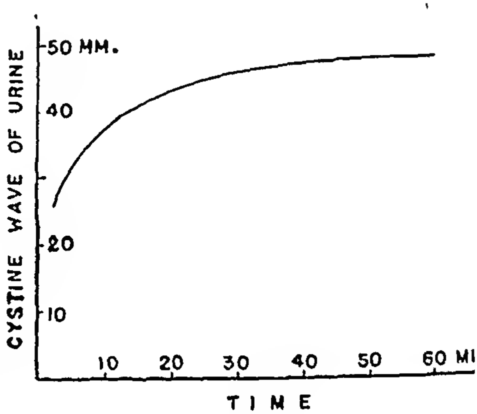

Fig. 2

Fig. 1. Effect of increasing concentrations of cystine on the polarographic wave.

Fig. 2. Effect of time on the height of the polarographic cystine wave of urine.

increase of wave height with increasing concentration was observed (Fig. 1).

The time factor in polarographic estimations of pure cystine solutions is negligible if oxidation of the cobaltous complex is prevented by keeping the solution in a glass-stoppered bottle prior to polarographic analysis. The height of the polarographic cystine wave does not change with time, whether cystine, cysteine, or protein hydrolysates are estimated. If, however, the polarographic record of 0.5 ml. of urine in 200 ml. of cobalt buffer is made at 5 minute intervals after the addition of urine, it is found

that the height of the cystine wave increases with time and reaches a maximum and constant value after 1 hour (Fig. 2). This indicates that the concentration of free cystine was increasing in the solution. A retarding effect of other urine constituents on the development of the cystine curve must be excluded, as added cystine can be recovered quantitatively and immediately. The same is true for cystine which was added to a synthetic urine (urine prepared according to Okuda with the addition of the oxyproteic acid fraction of a normal urine).

The original concentration of cystine in urine will be called "free" cystine. Its value may be obtained by extrapolation of the curve in Fig. 2 for the time zero. This value is analytically the least accurate. The cystine corresponding to its maximum concentration after 1 hour will be called "total" cystine and may be

TABLE I

Cystine Concentration in Urine in Mg. Per Cent

Method	Time	Unhydrolyzed	Hydrolyzed 5 hrs.	Hydrolyzed 18 hrs.
Polarographic	0	6-8		
	5 min.	13.6	15.3	17.0
	1 hr.	21.4	20.0	18.1
Colorimetric		7.3	17.1	19.2

estimated with an accuracy of ±5 per cent. The difference between "free" and "total" cystine will be called "liberated" cystine. The ammonia-ammonium chloride buffer or the cobalt chloride alone failed to free any cystine from urine. The liberating action must therefore be due to the formation of the cysteine-cobalt complex in ammoniacal solution.

The amount of free cystine excreted in 24 hours varied from 40 to 80 mg. in twenty healthy persons. These values agree well with those obtained by previous workers (1–4). The amount of total cystine varied from 100 to 200 mg.; hence the amount of liberated cystine varied from 60 to 120 mg. "The excretion of a cystine complex which decomposes in urine" has been reported by Brand *et al.* for cystinuric urine (7). Sullivan and Hess (4) reported an increase of from 35 to 100 per cent of the original concentration of cystine on acid hydrolysis of urine.

Eight urine samples from different persons were analyzed for

cystine with the polarographic and colorimetric procedure before and after hydrolysis. The colorimetric method of Sullivan and Hess (4) was used throughout the work because of its specificity for cystine. Transmittancies were read with the Coleman spectrophotometer at 5600 Å. Light absorption due to the yellow color of urine and the brown color of the hydrolysate was estimated in blank determinations and accounted for in the calculation of cystine values. Hydrolysis was carried out with concentrated hydrochloric acid according to Sullivan and Hess (4). Table I shows the result of a typical experiment and the degree of correlation existing between the two methods and suggests that acid hydrolysis liberates the same amount of cystine as the ammoniacal cobalt buffer. The nature of the substance from which cystine is liberated on hydrolysis could not be ascertained. The substance diffuses through cellophane membranes.

SUMMARY

1. From 40 to 80 mg. of free cystine are found polarographically in the 24 hour urine samples of twenty healthy persons.

2. The presence of a cystine complex in urine from which cystine is liberated by acid hydrolysis or treatment with ammoniacal cobalt buffers is demonstrated.

3. Polarographic and colorimetric values for cystine show correlation within the limits of error in unhydrolyzed and hydrolyzed urine.

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THE BIOCHEMICAL DEFECT IN CHOLINE-DEFICIENT RATS*

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The ability of the rat to synthesize choline is well established. Du Vigneaud *et al.* fed methionine containing deuterium in the methyl group, and found deuterium in the methyl groups of the choline fraction of the animals (1). Stetten observed that the N^{15} of ingested ethanolamine, and to a lesser extent of betaine, appeared in part as tissue choline (2). The action of choline in the prevention and cure of fatty livers (3) or hemorrhagic kidneys (4) has been duplicated by feeding betaine or methionine (5–8). The severity of renal lesions has been reduced by dietary creatine (9), and these latter observations have led to the concept of a requirement for "labile methyl groups" rather than for choline specifically (10). We have observed (11) that the choline content of growing rats may increase 4- or 5-fold on a choline-free diet, and that the choline content per gm. of rat tissue remains constant on diets of widely differing composition. These latter experiments were conducted over relatively long periods, so that any renal lesions which may have developed had time to regress. Since it is not clear whether renal lesions are due to a deficiency of choline itself or to a lack of some other methyl-containing essential, we have now determined the choline content of organs from animals which actually showed symptoms of "choline deficiency." Other experiments included a demonstration of choline synthesis during the critical period when symptoms developed, a determination of the choline content of rats on diets of variable methyl content, and a study of growth and reproduction on a synthetic diet free of choline.

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EXPERIMENTAL

Choline Content of Pathological Animals—Four pairs of rats, 35 to 40 gm. in weight, were fed the choline-free basal ration, Diet I (11), which consisted of extracted casein (12) 18, cerelose 73, Wesson salts (13) 4, and corn oil 5. In addition, each rat received the following supplements daily: 10 γ of carotene and 5 γ of calciferol dissolved in 1 drop of partially hydrogenated coconut oil, 20 γ of riboflavin, 10 γ of thiamine, 20 γ of pyridoxine, and 50 γ of pantothenic acid. Pair 1 received the control diet

TABLE I

Choline Content of Choline-Deficient Rats

Pair No. and diet	Weight of rat	Kidney hemorrhage	Total choline					
			Kidney	Liver	Brain	Carcass	Total	Per gm. rat
	gm.		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1. Diet I (basal)	35	Severe	1.4	4.7	4.4	28.0	38.5	1.10
	40		1.5	5.3	4.2	35.0	46.0	1.15
2. " " + 25 mg. ethanolamine per rat per day	35	Very severe	1.4	6.0	3.4	28.9	39.7	1.13
	41		1.5	5.8	3.7	41.1	52.1	1.27
					Brain + carcass			
3. Diet I + 0.33 mg. choline per rat per day	43	Slight	1.0	2.9	38.3		42.2	0.98
	47		1.0	2.2	46.3		49.5	1.05
4. Diet I + 5 mg. choline per rat per day	40	None	1.1	2.4	40.8		44.3	1.11
	42		0.9	2.4	42.7		46.0	1.10

only; Pair 2 received an additional 25 mg. of ethanolamine daily per rat; Pair 3, 0.33 mg. of choline daily per rat; and Pair 4, 5 mg. of choline daily per rat. The rats were killed at the end of 7 days and the degree of kidney hemorrhage evaluated.[1] The kidneys, liver, brain, and carcass of each animal were then analyzed separately for choline by our modification of the reineckate method previously described (11). For Pairs 3 and 4 the brains were included with the carcasses.

[1] We are indebted to Mr. R. M. Johnson and Dr. F. W. Quackenbush for these animals.

The results are presented in Table I. Contrary to what one might expect in a true choline deficiency, the choline content of rats with hemorrhagic kidneys was not reduced but rather was slightly above normal. The rats in Pairs 1 and 2, all with kidney lesions, contained an average of 1.15 mg. of choline per gm. of rat, ranging from 1.10 to 1.27 mg. Pair 4, fed 5 mg. of choline daily, averaged 1.10 mg. of choline per gm. of rat. The average for rats of comparable weight raised on our stock ration is 1.03 mg. per gm. (0.90 to 1.14 mg.) and for mature rats, 0.82 mg. (0.79 to 0.84 mg.) (11). When the control diet was supplemented with choline in the present series, the amount of choline in the liver and kidneys decreased somewhat. Thus for Pairs 1 and 2, the choline content averaged 5.5 mg. per liver and 1.45 mg. per pair of kidneys. The livers and kidneys of rats (Pairs 3 and 4) which received choline averaged 2.5 and 1.0 mg. respectively. In the case of liver the difference in choline content was more than a reflection of a change in size of the organ; pathological livers contained an average of 2.1 mg. of choline per gm., whereas after the administration of choline the content was 1.5 mg. per gm. However, the choline content per gm. of kidney was not altered by feeding choline; *i.e.*, the increased choline content in pathological kidneys paralleled their increased size.

The slightly increased choline content per gm. of pathological animals was confirmed in a second series of rats fed a high fat diet. Diet II had the following composition: crude casein 18, sucrose 38.9, lard 30, Wesson salts 5, brewers' yeast 8, irradiated yeast 0.1. Each rat received 50 γ of carotene in 1 drop of lard weekly. The rats ranged from 39 to 47 gm. in weight. Notwithstanding the fact that some choline was present in the diet, typical deficiency symptoms developed in many of the animals. Hence on the 9th day five sick rats of each sex were killed for analysis as well as five apparently normal females. The gastrointestinal tract of each rat was removed before analysis.

The normal animals averaged 0.78 mg. of choline per gm. of rat (Table II); the sick females averaged 0.94 mg. and the sick males, 0.99 mg. per gm. Since the ranges of values for the normal (0.74 to 0.85 mg. per gm.) and the pathological groups (0.88 to 1.10 mg. per gm.) did not overlap, the differences were considered significant. The results of the two series therefore suggest that

the biochemical defect in the rats with hemorrhagic kidneys is not in the choline content but rather in some other methylated essential.

Griffith (14) observed that the symptoms of choline deficiency appeared more slowly and were less severe in young female rats than in male rats of the same age and weight. This was confirmed with our high fat diet in the previous experiment. Hence it was of interest to determine whether this sex difference was a reflection of differences in choline content in very young rats. Accordingly four females and four males 27 days of age and 36 to 45 gm. in weight were analyzed for choline. The males contained from 1.06 to 1.14 mg. of choline per gm. of rat, average 1.10 mg.; the females contained 0.95 to 1.06 mg. of choline per

TABLE II

Choline Content of Choline-Deficient Male and Female Rats

Sex	Condition of rat	Condition of kidneys	Weight of rat	Total choline	Choline per gm. rat
			gm.	*mg.*	*mg.*
Female	Normal	Normal	69	51.5	0.75
			66	56.0	0.85
			65	49.2	0.76
			63	46.8	0.74
			66	51.5	0.78
Average......				51.0	0.78
Female	Inactive, weak	Hemorrhagic	49	45.5	0.93
			48	44.3	0.92
			41	36.1	0.88
			45	45.9	1.02
			45	42.5	0.95
Average......				42.9	0.94
Male	Inactive, weak	Hemorrhagic	49	48.7	1.00
			54	50.3	0.93
			38	35.6	0.94
			48	53.0	1.10
			47	45.2	0.96
Average......				46.6	0.99

gm., average 1.01 mg. In other words the young female rats did not possess any greater stores of choline than did the males.

Choline Synthesis During Critical Period—Griffith has noted that kidney lesions readily occur in rats 20 to 30 days of age on a low choline diet (14), but that there is a marked decrease in the incidence of lesions when rats 33 days of age or older are fed the deficient diet. In the younger rats the lesions were most severe at 7 to 10 days, but by 14 days considerable healing had occurred (14). Since older rats possess both the power to synthesize choline and to prevent and cure kidney lesions, the development of lesions in younger animals might be attributable to a failure in choline synthesis during the critical period. This possibility was

TABLE III

Choline Content of Rats at End of Critical Period

Diet	Weight of rat	Total choline					
		Kidney	Liver	Brain	Carcass	Total	Per gm. rat
	gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Low choline, Diet I	73	1.1	6.7	3.4	56.9	68.1	0.93
	80	2.3	8.3	4.9	64.4	79.9	1.00
" " low fat, Diet III	66	1.5	6.8	4.7	50.8	63.8	0.97
	67	1.1	6.5	4.0	54.3	65.9	0.98
	66	1.4	8.8	4.3	51.2	65.7	0.99

investigated as follows: Six rats, 21 days of age and 36 to 41 gm. in weight, were divided into two equal groups. Group 1 received the choline-free basal Diet I described above; Group 2 received a low fat, choline-free Diet III consisting of extracted casein 18, cerelose 78, and Wesson salts 4. The diets were supplemented with 4 drops of choline-free rice bran concentrate (11), 20 γ of riboflavin, 10 γ of thiamine, 10 γ of carotene, and 5 γ of calciferol in 1 drop of partially hydrogenated coconut oil daily per rat. The rats were killed at the end of 14 days and the kidneys, liver, brain, and carcass of each rat analyzed separately for choline.

All rats survived the experimental period except one in Group 1 which died on the 7th day with kidney lesions. After 14 days on the low choline rations the rats contained an average of 69 mg. of choline per animal (Table III). Rats 20 to 30 days of age

contain an average of only 43 mg. of choline per animal (11). Thus approximately 26 mg. of choline had been synthesized by each rat during the critical 14 day period, and the choline content per gm. of rat remained normal. Incidentally, choline synthesis was not altered by the diet very low in fat.

Diets Very Low in Labile Methyl Group—Our previous choline-free diets were all relatively high in labile methyl groups in the form of methionine; a diet low in methyl groups was realized by replacing the casein of Diet I with arachin (15), which contains only approximately 0.5 per cent of methionine (16). Diets containing zein as the source of protein were also fed. Thus the effect of methionine deficiency was compared with an amino acid deficiency not involving methyl groups. Both diets were fed with and without added choline.

Twelve rats, 90 to 105 gm. in weight, were divided into four comparable groups. Group 1 received the low methionine, choline-free diet consisting of arachin 18, cerelose 73, corn oil 5, and Wesson salts 4. Group 2 received the same diet plus 24 mg. of choline per rat per day. Groups 3 and 4 received the diets of Groups 1 and 2, respectively, except that zein was fed as a source of protein in place of arachin. Choline-free rice bran concentrate, flavin, thiamine, carotene, and calciferol were administered in the usual amounts. The experiment was discontinued at the end of 5 weeks.

Both groups receiving arachin showed an initial loss in weight during the first 2 weeks. However, Group 2 receiving choline lost only 17 gm., whereas Group 1 lost 29 gm. After the 2nd week both groups resumed growth. The rate of growth was much greater for the group receiving choline, so that at the end of the experiment this group weighed on an average 22 gm. more than the control group which had received no choline. Both groups receiving zein lost an average of 36 gm. during the 5 week experimental period. In other words, choline appears to have exerted a sparing effect on the small amounts of methionine in the arachin diets, but was without effect on the deficiencies in the zein diets.

In this connection it was of interest to determine whether choline would prevent kidney hemorrhage in young rats on the diet low in labile methyl. Eighteen rats, 35 to 40 gm. in weight, were

divided into six equal groups comparable as to age, sex, and weight. Group 1 received the basal arachin diet described above. Groups 2 and 3 received the same diet as Group 1 plus 1 mg. of choline per rat daily and 4.4 mg. of choline per gm. of ration (about 20 mg. of choline per day), respectively. Group 4 received the choline-free basal ration Diet I in which casein was the source of protein. Groups 5 and 6 received the same diet as Group 4 plus 2 mg. of choline (about 10 mg. daily) or 38 mg. of sarcosine (about 190 mg. daily) per gm. of ration, respectively. All the diets were supplemented with 20 γ of riboflavin, 10 γ of thiamine, 100 γ of pantothenic acid, 30 γ of pyridoxine, 10 γ of carotene, and 5 γ of

TABLE IV

Severity of Kidney Degeneration in Young Rats on Various Diets

Group No.	Diet	Description of kidneys		
		Rat 1	Rat 2	Rat 3
1	Arachin	++++	++++	++
2	" + 1 mg. choline per rat per day	Normal	Normal	Normal
3	Arachin + 4.4 mg. choline per gm. ration (*ca.* 20 mg. choline per day)	"	"	"
4	Casein	++++	++	++
5	" + choline	Normal	Normal	Normal
6	" + sarcosine	++++	++++	++

calciferol. The rats were killed after 10 days and the degree of kidney hemorrhage recorded.

In agreement with others (17) typical severe hemorrhagic kidneys resulted on the arachin diet in the absence of choline (Table IV), even though the rats lost an average of 4 gm. during the experimental period. Griffith and Mulford have reported that animals which failed to grow due to an inadequate food intake failed to develop hemorrhagic kidneys (10). On the arachin diet, however, the need for methyl groups was apparently so acute that lesions developed in animals which were losing weight. The addition of choline to the arachin diet prevented kidney lesions completely, although it did not prevent a loss in weight. In other words choline was effective in the absence of adequate dietary

methionine, thus eliminating methionine as the methyl compound whose absence causes kidney lesions. Of incidental interest was the observation that sarcosine, an N-methyl compound, did not protect against kidney hemorrhage. The methyl group of sarcosine is thought to be removed as formaldehyde (18) and hence would not contribute to the labile methyl supply.

The effect of the arachin diet on the choline content of rats was determined as follows: Four rats, 66 to 72 gm. in weight, were fed the arachin diet for 16 days, when they were killed, and the kidneys, liver, brain, and carcass of each analyzed individually for choline. The rats declined in weight at a rate of 1 gm. per day, and the livers at autopsy appeared very fatty. Nevertheless the analytical results revealed that the choline content remained proportional to body weight, an average of 0.81 mg. of choline being present per gm. of rat at the end of the experiment. The choline content per gm. of liver, however, was decreased. An average value of 1 mg. (range 0.7 to 1.2 mg.) was obtained as contrasted to the normal level of approximately 2.3 mg. per gm. (11).

Selenium was fed in an indirect attempt to lower the labile methyl supply, since in selenium toxicity there is a garlic-like odor (19) which is ascribed to methyl selenide (20). Thus it appeared possible that selenium might function as a methyl acceptor, withdrawing methyl groups from other compounds and excreting them. In Series I, eight rats, 50 to 76 gm. in weight, were divided into two equal groups comparable as to age, sex, and weight. One group received the choline-free basal Diet I plus 17.5 parts per million of Se as Na_2SeO_3. The other group received the same diet plus 0.3 per cent choline. Each diet was supplemented with 4 drops of choline-free rice bran concentrate, 20 γ of riboflavin, 10 γ of thiamine, 10 γ of carotene, and 5 γ of calciferol in 1 drop of partially hydrogenated coconut oil daily per rat. After 4 weeks two rats from each group were analyzed for choline. In Series II, eight rats, 110 to 162 gm. in weight, were divided into two comparable groups. One group received stock ration (21) plus 70 parts per million of Se as Na_2SeO_3. The other group received the same diet plus 0.5 per cent choline. After 4 weeks the livers from two rats in each group were analyzed for choline.

The addition of selenite to the diet did not change the choline

content of the rats. In Series I, those rats which received no choline averaged 1 mg. of choline per gm. of rat (0.94 to 1.07 gm.); those rats which received choline averaged 0.97 mg. per gm. (0.89 to 1.05 gm.). In Series II, the choline content of the livers of the two groups receiving Se with and without choline were essentially normal. Moreover, choline had no effect on the course of Se toxicity. The rats in Series I gained an average of 4 gm. in the experimental period of 4 weeks. The rats in Series II showed an average total weight loss of 25 gm. The latter rats also had the characteristic garlic-like odor and cirrhosis of the livers. Evidently the amount of labile methyl group necessary for a detectable odor of methyl selenide represents only a very minor fraction of the total methyl supply.

Growth and Reproduction on a Diet Free of Choline—Jukes (22) raised two generations of rats on a synthetic diet in which the well characterized members of the vitamin B complex were supplied in crystalline form. His diet contained 50 mg. of choline per 100 gm. of ration. In view of the fact that the rat synthesizes choline, and that an abundant supply of labile methyl group was present in the Jukes ration as methionine, it appeared possible that the choline could be omitted without serious consequences if the young rats survived the critical period. This was done in the following experiment. Fifteen rats, 36 to 46 gm. in weight, were divided into two groups comparable in age, sex, and weight. Group 1 (eight rats) received a choline-free diet consisting of sucrose 65, extracted casein 25, corn oil 5, and Wesson salts 5. The following vitamin supplements were added per 100 gm. of ration: 1 mg. of carotene, 0.5 mg. of calciferol, 2.8 mg. of pantothenic acid, 1.0 mg. of nicotinic acid, 0.5 mg. of riboflavin, 0.2 mg. of thiamine, and 0.2 mg. of pyridoxine. Group 2 (seven rats) received 50 mg. of choline per 100 gm. of ration in addition to the above supplements. Three of the rats in each group were females. With the exception of one rat in Group 1, which died with kidney hemorrhage on the 8th day of the experiment, all grew to maturity. The rats receiving choline (Group 2) grew at a slightly greater initial rate, but after the 2nd week both groups showed essentially the same rate of growth (Table V). After the 5th week of the experiment (9th week of life), the growth rate in Group 2 declined, whereas in

Group 1 it continued undiminished for 4 weeks more. As a result the rats in both groups were of essentially the same average weight at the end of 19 weeks; *i.e.*, 270 gm.

Two females in Group 1 and one female in Group 2 gave birth to litters of six, five, and six, respectively. At 7 weeks of age, the average weight per rat in each litter was 81, 89, and 96 gm., respectively. Thus in the second generation as in the first, the rate of growth was somewhat less when choline was omitted from the diets, but that the young survived at all indicates the capacity of the rat to synthesize choline.[2]

TABLE V

Growth of Rats on Synthetic Diet Free of Choline

Age	Average weekly weight gains				
	Group 1. No choline	Group 2. Choline	Litter 1. No choline	Litter 2. No choline	Litter 3. Choline
wks.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1			5	3	4
2			5	7	7
3			4	9	7
4	12	18	6	10	13
5	13	23	11	19	19
6	26	28	13	12	14
7	17	20	14	9	13
8	21	32	18		
9	27	9	14		
10	20	9	16		
11	21	15			
12	11	13			

DISCUSSION

In spite of the well known action of choline in preventing kidney lesions in rats, it does not appear that the missing agent in the pathological animals is choline itself. Nor does the essential compound appear to be methionine, for in our experiments choline completely prevented hemorrhagic kidneys on a diet containing amounts of methionine insufficient for growth, and furthermore homocystine, which yields methionine on methylation, is reported

[2] Both in the presence and absence of choline, rats of both generations developed a condition of "spectacle eye" (23), indicating that the original Jukes diet was probably not complete in all essential dietary factors.

to aggravate kidney hemorrhages (6). The essential compound most probably contains a labile methyl group.

One possibility is that a particular choline complex is missing; another is that an oxidation product of choline might be involved. Indeed several observations (2, 6, 21, 25) can be rationalized if the assumption is made that methyl groups are released by an oxidation product rather than by choline itself. The physiological rôle of choline oxidase has never been established, and it may be of significance that the highest concentrations of the enzyme are found in liver and kidney (26), the two tissues which show the most obvious sensitivity to choline deficiency. The enzyme is markedly inhibited *in vitro* by stearic acid (27). Since fatty livers not only accompany hemorrhagic kidneys but definitely precede them (10), an observation which we have been able to confirm, it is not unreasonable to postulate a connection between the abnormalities in the two organs. Thus the development of kidney lesions might be pictured as follows: From the 20th to the 33rd day of life, a large amount of choline is needed by the rat for structural and lipotropic purposes, and there is also a need for other methylated essentials. In the absence of an adequate supply of methyl groups, the amount of choline synthesized is insufficient for normal fat transport, and hence fat accumulates in the liver. The presence of this extra fat inhibits choline oxidase, so that the methyl groups in the choline become less available for methylation purposes owing to a failure in the necessary preliminary oxidation mechanism. Thus a deficiency in other methyl-containing compounds results, including the unknown needed for the maintenance of normal kidney structure.

SUMMARY

The choline content of rats with symptoms of "choline deficiency" was not reduced but was slightly greater than normal. Hemorrhagic kidneys contained somewhat more choline than normal kidneys. No sex difference was observed in the choline content of young rats. Moreover, choline synthesis appeared to proceed normally during the critical period when deficiency symptoms develop. It is concluded that symptoms of "choline deficiency" are due to the lack of a methyl-containing essential other than choline itself.

The choline content of rats remained essentially normal on diets

low in "labile methyl," diets low in fat, or diets containing sodium selenite. Young rats on a diet low in methionine declined in weight but nevertheless developed kidney hemorrhage which could be prevented by choline. Rats grew to maturity and reproduced on a synthetic diet free of choline.

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STUDIES ON ANEMIA IN DOGS DUE TO PYRIDOXINE DEFICIENCY*

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Nutritional microcytic anemia in puppies was first reported by Fouts *et al.* (1) on a synthetic ration low in pyridoxine. The anemia was curable by concentrates and later by crystalline pyridoxine (2). These authors showed that adult dogs placed on such a ration also developed the condition (3). These results have been confirmed in the authors' laboratory (4) and further studies have been conducted by Borson and Mettier (5).

In connection with our studies on blood formation in dogs we became interested in the metabolism of iron and copper in an anemia due entirely to deficiency of an organic factor. We have therefore undertaken a study of the plasma iron and total blood copper levels of the blood of pyridoxine-deficient dogs during both the anemia and the remission. Previous experience in our laboratory and in other laboratories with this anemia had indicated that perhaps other factors were involved in blood formation, since slow incomplete remissions had been frequently observed. It was thought that this experiment might throw some light on the nature of such a factor.

EXPERIMENTAL

Five mongrel puppies from four different litters and two adult dogs from different litters were used in these experiments. They

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the Wisconsin Alumni Research Foundation. We are indebted to the Works Progress Administration for assistance in the care of the animals and to Merck and Company, Inc., Rahway, New Jersey, for generous supplies of thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline.

were placed on a highly purified ration which we have used extensively in our vitamin B complex studies with dogs (4). It has the following per cent composition: sucrose 63, purified casein 19, cottonseed oil 8, cod liver oil 3, Salts 3[1] 4, liver extract fraction 3.

The liver extract used was Fraction B of The Wilson Laboratories containing about 25 γ of pyridoxine per gm. This fraction was treated once with English fullers' earth to lower its pyridoxine content. The ration was supplemented with 100 γ per kilo of body weight per day of thiamine and riboflavin, 2 mg. per kilo of nicotinic acid, and 400 γ of calcium pantothenate per kilo per day. These vitamins were administered orally in aqueous solution twice weekly. Later on, when the importance of choline was recognized (7), this was included at a level of 50 mg. per kilo per day.

Since the microcytic anemia is characteristic of chronic pyridoxine deficiency, it was frequently necessary to prevent the onset of the acute deficiency by administration of small quantities of crystalline vitamin B_6. This procedure was, however, unnecessary after the first 2 months. Blood samples were collected usually at weekly intervals from the radial vein. Hemoglobin determinations were done in duplicate according to the method of Evelyn (8); hematocrit readings were obtained with Wintrobe tubes. Plasma iron determinations were made on filtrates of blood plasma from a hot trichloroacetic acid precipitation. The filtrates were treated with thioglycolic acid and the ferrous iron then reacted with α,α'-dipyridyl (pH 5.4). The resulting color was then determined on the Evelyn photoelectric colorimeter. This method is to be published in detail in the near future. Blood copper determinations were made on a low temperature ash 425°) of the dried blood in vitreosil dishes. The copper in the ash was determined by the diethyldithio carbamate method of Coulson (9). Amyl alcohol was used for the color extraction and the extracts were filtered through No. 40 quantitative filter paper to remove all traces of water. 12 gm. of blood were used for each determination and two recovery samples were used in each set of determinations. Recoveries ranged from 76 to 100

[1] Salts 3 is the same as Salts 1 (6) with an additional 1.21 gm. of $MnSO_4 \cdot 4H_2O$ per kilo of salt mixture.

TABLE I

Blood Chemical and Hematological Data from Five Dogs on Pyridoxine-Deficient Ration

Dog No.	Day	Weight	Hemoglobin	Hemoglobin change	Hematocrit	Red blood cells	Mean cell volume	Saturation index*	Plasma Fe	Blood Cu
		lbs.	gm. per cent	gm.	per cent	millions per c.mm.	c. μ		γ per cent	γ per cent
184	36	3.45	10.06		34.5	6.42	54	0.292		
	83	6.1	9.34	19.2	31	5.67	54.5	0.301	106	
	124	6.0	7.43	−3.2	24.5	5.26	46.5	0.303	304	49
	134	5.7	6.59	−2.6	24	5.29	45.5	0.274	271	164
	Started 100 γ pyridoxine hydrochloride per kilo per day									
	151	7.2	9.96	32.4	34	5.42	63	0.293	111	122
	166	7.75	9.34	6.5	32.5	4.82	67.5	0.287	122	106
	Started 250 mg. inositol per day									
	190	8.3	10.12	15.9	35	5.19	67.5	0.290	228	
	Started 6% liver extract powder									
	224	10.0	13.69	51.9	43.5	6.66	65.5	0.315	94	
190	33	7.15	11.37		38	6.66	57	0.299		
	79	10.35	7.04	−2.9	22.5	4.51	50	0.313	230	
	106	10.05	4.08	−18.6	16.5	3.28	50.5	0.248	356	80
	Started 100 γ pyridoxine hydrochloride per kilo per day									
	113	11.60	9.26	54.6	34.5	5.46	63	0.268	85	146
	139	14.00	10.85	47.5	34.5	5.86	58.5	0.317	76	120
	Started 8% whole liver substance									
	188	15.20	11.55	29.3	37	5.65	65.5	0.312	97	115
	Started 8% rice bran extract									
	220	15.65	12.56	27.2	38.5	6.18	62.5	0.326	90	206
	Started 6% liver Fraction D									
	234	15.90	13.34	17.3	43	7.16	60	0.311	79	144
191	33	5.35	11.28		37.5	5.53	68	0.301		
	65	8.15	10.58	24.4	33	7.16	46	0.321	161	
	85	9.50	9.47	7.0	30.5	6.37	48	0.311	234	
	96	9.85	9.34	6.3	31	6.91	45	0.301	157	158
	122	10.20	6.24	−17.3	21.5	5.27	41	0.290	360	
	134	10.23	5.23	−7.0	18.5	4.33	42.5	0.283	398	89
	Started 100 γ pyridoxine hydrochloride per kilo per day									
	142	11.20	10.12	49.9	35	6.72	51.5	0.292	77	125
	150	12.00	9.47	4.1	36	6.53	55	0.263	63	123
	159	13.10	10.93	27.4	35	5.62	62.5	0.313	85	102
	171	12.90	11.67	9.9	36.5	6.41	57	0.320	93	
	187	13.50	11.28	8.5	38	6.87	55.5	0.297	88	134
	196	13.95	11.71	13.6	37	6.58	56	0.317	58	
	205	13.90	11.98	4.4	36.5	6.35	57.5	0.328	73	

TABLE I—*Concluded*

Dog No.	Day	Weight	Hemoglobin	Hemoglobin change	Hematocrit	Red blood cells	Mean cell volume	Saturation index*	Plasma Fe	Blood Cu
		kg.	*gm. per cent*	*gm.*	*per cent*	*millions per c.mm.*	*c. μ*		*γ per cent*	*γ per cent*
	Started 6% liver extract Fraction D									
	219	14.20	13.17	21.2	39.5	6.31	62.5	0.334	89	
99	90	7.80	6.91		23.5	4.01	58.5	0.294	366	
	120	7.40	5.81	−6.8	21.5	4.31	50	0.270	245	84
	144	7.60	4.62	−4.3	17	3.81	44.5	0.272	382	84
	Started 100 γ pyridoxine hydrochloride per kilo per day									
	162	8.30	9.65	39.7	34	5.66	60	0.284	154	114
	186	8.00	10.97	13.4	37.5	6.51	57.5	0.292	108	
	Started 500 γ cobalt per day									
	202	8.00	11.83	11.5	36.5	6.20	59	0.325	108	
	Started 8% yeast									
	218	7.95	11.37	0.5	37	6.30	59	0.308	89	116
	Started 5% Pb and Hg filtrate of liver Fraction D									
	245	7.75	12.17	8.9	38.5	6.27	61.5	0.316	114	131
208	106	6.2	6.48		23.5	5.17	45.5	0.276	200	109
	Started 100 γ pyridoxine hydrochloride per kilo body weight per day									
	113	7.25	9.92	27.4	35	6.69	52.5	0.283	206	
	125	8.2	9.84	8.8	32.5	5.08	64	0.303	88	
	Increased calcium pantothenate to 1.1 mg. per kilo body weight per day									
	139	9.48	9.96	12.9	32	5.15	62	0.311	150	
	Started 6% liver Fraction D on ration									
	152	11.3	11.21	27.7	38.3	5.19	74	0.293	115	121
	169	12.15	12.14	22.9	38	5.44	70	0.320	139	

* Measured in gm. of Hb per cc. of packed cells.

per cent. Since rather large quantities of blood were required for the copper determinations, they were not carried out routinely but at chosen intervals.

The results so obtained from Dogs 99, 190, 184, 191, and 208 are given in Table I; those from Dog 182 are shown in Fig. 1. For the sake of brevity many of the data from the dogs have been omitted except in the case of Dog 191 for which results are presented in some detail. In evaluation of the hemoglobin response to the various supplements it was necessary to minimize errors

arising from fluctuations in weight. Assuming 8 per cent of body weight as blood, the total body hemoglobin was calculated from the hemoglobin level of the blood. These values, together with the known amount of hemoglobin removed as the analysis sample, permitted the calculation of body "hemoglobin change" given in Table I. The figures given are for the period from the previous to the present analysis.

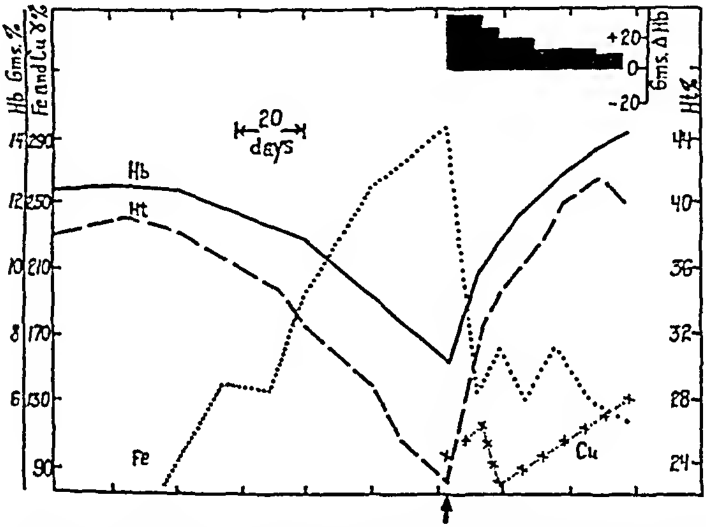

Fig. 1. Blood changes in Dog 182 following supplementation of the basal pyridoxine-deficient ration with 100 γ of pyridoxine hydrochloride per kilo of body weight per day and 3 per cent liver extract powder. Therapy was initiated at the point indicated by the arrow.

The dogs all developed the characteristic deficiency anemia within $3\frac{1}{2}$ to $4\frac{1}{2}$ months. The two adult dogs (Nos. 99 and 110) were bled while on the deficient ration in order to hasten the deficiency. Only data from Dog 99 will be reported in this paper. The blood hemoglobin, hematocrit, red cell count, and mean cell volume values in the anemic dogs were comparable in all respects to those reported by other investigators. The addition of 100 γ per kilo of body weight per day of pyridoxine hydrochloride resulted in an immediate and rapid generation of red blood cells. However, after a hemoglobin level of 10 to 11 gm. per cent was reached, there were no further appreciable increases

in the hemoglobin, hematocrit, and red cell count. The plasma iron level, abnormally high during the anemia, fell to a low normal level. In contrast to this picture Dog 182 was given 3 per cent liver extract powder 1:20 (The Wilson Laboratories) in place of the basal liver fraction at the time when the pyridoxine therapy was started. The blood values in this dog rose steadily to approach normal figures.

Other supplements were tried on the rest of the dogs in an attempt to stimulate blood formation to more normal hemoglobin levels. From the results it can be seen that the response to all of these supplements was much less than to the original administration of pyridoxine. Of the supplements tried, liver extracts seemed to be the most effective but relatively high levels were used and several weeks were necessary to produce normal values. In single trials rice bran extract (vitab, Type II)[2] and yeast (Northwestern) seemed less effective, as did inositol and cobalt.

DISCUSSION

Failure to demonstrate a complete remission from the anemia due to pyridoxine deficiency has been observed by Borson and Mettier (5) when pyridoxine was added to a ration of this type. Fouts *et al.* (1–3) found rapid and complete remissions in nearly all their dogs when pyridoxine was added to their ration. The differences in the vitamin B complex sources used must be advanced as the probable explanation for these differences in response. Since the former authors found that their fullers' earth filtrate of liver extract would promote further blood formation if included in the ration at higher levels, they suggested that the "filtrate factor" was also involved in blood formation. The identity of this filtrate factor could not be surmised from their studies. Our results obtained with Dog 208 would seem to indicate that this factor is neither pantothenic acid nor choline. After the plateau in the blood hemoglobin level was reached, synthetic calcium pantothenate was added to the diet to increase the daily intake to 1100 γ per kilo per day over the period of treatment. The failure to respond to this and the subsequent response to liver extract Fraction D (The Wilson Laboratories) indicates that another substance is involved.

[2] Kindly furnished us by the National Oil Products Company, Harrison, New Jersey.

The failure of Dog 99 to respond to the lead and mercury filtrate fraction of Fraction D may be insignificant in the light of the necropsy performed immediately after the completion of the experiment. The animal appeared normal except for a greatly enlarged spleen which weighed 82 gm. when first removed and measured $8\frac{1}{2}$ inches in length. Considering the size of the animal, it would seem to be from 4 to 8 times the normal size. The condition of this organ which is so intimately concerned with the regulation of certain elements of the blood is interesting in view of the long history of experimental anemia in this animal.

The iron levels in blood plasma progressively increase as the anemia becomes more severe. The extremely high values of 398, 310, 356, 304, 382, and 310 γ per cent of plasma obtained with six of the seven dogs in this study compare favorably with the value of 476 γ found by Fouts *et al.* (3). The high level of iron in the plasma suggests an attempt by the organism to mobilize those materials necessary for blood formation that they may be available to the hematopoietic tissues. It is evident that vitamin B_6 deficiency does not impair absorption of iron from the intestine nor would it appear to interfere with the mobilization of iron. During the remission from the extreme anemia of pyridoxine deficiency and the subsequent slowing of blood formation, the plasma iron drops to a low normal level. These levels are comparable to those observed in our hemorrhagic anemia studies (10) in which a mineralized milk ration was used. In the latter studies, however, remission from the anemia was always accompanied by striking increases in the plasma iron level, while this was not observed with liver therapy in these dogs. Nevertheless, it is more than possible that the rise afforded by liver extract in both these studies is due to the same hematopoietic stimulant.

The blood copper levels did not follow as consistent a pattern as did the plasma iron. In general, the severely anemic dogs had a blood copper level of 80 to 100 γ per cent which rose slightly to 100 to 140 γ per cent after treatment with pyridoxine. The levels of blood copper in the anemic animals are therefore of a low normal magnitude and thus pyridoxine deficiency would not appear to interfere with the absorption or metabolism of copper. It is interesting that, unlike iron, copper does not appear to be mobilized in the blood of the severely anemic animal.

The blood plasmas of Dogs 99 and 110 showed no detectable

bilirubin either during the anemia or after pyridoxine was administered. Bilirubin was determined by the method of Malloy and Evelyn (11) but the determination was not applied to the plasmas of the younger dogs.

SUMMARY

1. The blood plasma iron is abnormally high in anemia due to pyridoxine deficiency in dogs. It drops to a low normal level during the remission with pyridoxine therapy.

2. Total blood copper values are at a low normal level during the anemia and increase to normal during pyridoxine therapy.

3. Following the immediate stimulation in blood formation afforded by pyridoxine therapy, there is a lag which may be overcome by addition of liver extract to the ration. This stimulation is apparently not due to thiamine, riboflavin, nicotinic acid, pantothenic acid, or choline.

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THE SUCCINOXIDASE SYSTEM IN RIBOFLAVIN-DEFICIENT RATS*

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Thus far none of the enzymes which reduce cytochrome *c* has been isolated from animal tissues. Nevertheless the belief is held that coenzymes I and II are linked to cytochrome by flavoproteins (1, 2) and it has been suggested by Potter (1, 3) that succinic dehydrogenase may be an analogous enzyme. The recent demonstrations by Axelrod and coworkers (4, 5) that the concentrations of the flavoproteins, *d*-amino acid oxidase and xanthine oxidase, are decreased in the tissues of riboflavin-deficient rats suggested testing the hypothesis that succinic dehydrogenase is a flavoprotein by studying the concentration of this enzyme under conditions similar to those previously employed (4, 5). A positive result, while not establishing the point, would constitute *prima facie* evidence that the enzyme is a flavoprotein, and would at the same time give added significance to the dietary rôle of riboflavin, since the oxidation of succinic acid is believed to be an essential step in normal carbohydrate metabolism.

EXPERIMENTAL

Weanling male, albino rats were placed on an experimental diet which was designed to produce an uncomplicated riboflavin deficiency.[1] The ration was fed *ad libitum* unless designated otherwise.[2]

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† Jonathan Bowman Cancer Research Fellow.

[1] The composition of the diet (designated as Ration B) and the general behavior of the rats are fully described in a previous publication (4).

[2] We are indebted to Merck and Company, Inc., Rahway, New Jersey, for generous supplies of thiamine, pyridoxine, riboflavin, pantothenic acid, choline, and nicotinic acid, and to the Abbott Laboratories, North Chicago, Illinois, for the haliver oil used in the present study.

After a depletion period of 10 weeks, fifteen of the rats were sacrificed for the succinoxidase determinations, while the remainder were grouped as indicated in Table I and riboflavin therapy was instituted. These rats were sacrificed at the completion of their respective periods of therapy.

The succinoxidase content of the tissues was determined according to the method of Potter (3) with the following modifications: (*a*) heart and thigh muscles were minced in an apparatus described by Seevers and Shideman (6) previous to homogenization; (*b*)

TABLE I

Effect of Riboflavin Deficiency upon Succinoxidase Content of Rat Tissues

Group No.	No. of rats	Riboflavin* therapy	Succinoxidase activity† Liver	Kidney cortex	Brain	Heart muscle	Thigh muscle
I	15	None	64 (54– 75)	99 (86–122)	32 (18–41)	137 (119–163)	25 (22–33)
II	5	100 γ daily, 12 days	79 (75– 82)	113 (104–132)	35 (31–40)	158 (135–178)	35 (26–39)
III	10	100 γ daily, 12 days	95 (80–112)	104 (76–118)	33 (20–38)	150 (136–172)	30 (22–36)
IV	3	20 mg. per kilo body weight, 3 days	94 (80–106)	115 (113–118)	29 (25–35)	130 (106–144)	27 (24–30)

* After riboflavin therapy was begun, the food intake of each of the rats in Groups III and IV was restricted to 4 gm. of basal ration per day, which is the average daily food consumption by rats in the basal group. Therapy was administered orally to animals in Groups II and III and by subcutaneous injection to those in Group IV.

† The results are reported in terms of the Q_{O_2}; *i.e.*, the oxygen uptake per mg. of dry tissue per hour. The range of values is given in parentheses.

the reaction was carried out at 38°; (*c*) 20 γ of calcium (as calcium chloride) were added to each flask. The addition of calcium was considered necessary, since Axelrod, Swingle, and Elvehjem (7) have demonstrated the marked stimulatory effect of calcium upon the succinoxidase activity of various tissues. 20 γ of calcium produced the maximal effect in the tissues studied. As a routine procedure, the succinoxidase activity of each tissue was determined at levels of 2 and 3 $\times$ 10^{-8} mole of added cytochrome *c* Since the plateau level was reached at a cytochrome *c* concentration of 2 $\times$ 10^{-8} mole per flask, the two determinations served as

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The succinoxidase content of the tissues was determined according to the method of Potter (3) with the following modifications: (*a*) heart and thigh muscles were minced in an apparatus described by Seevers and Shideman (6) previous to homogenization; (*b*) the reaction was carried out at 38°; (*c*) 20 γ of calcium (as calcium chloride) were added to each flask. The addition of calcium was considered necessary, since Axelrod, Swingle, and Elvehjem (7) have demonstrated the marked stimulatory effect of calcium upon the succinoxidase activity of various tissues. 20 γ of calcium produced the maximal effect in the tissues studied. As a routine procedure, the succinoxidase activity of each tissue was determined at levels of 2 and 3 $\times$ 10^{-8} mole of added cytochrome *c* Since the plateau level was reached at a cytochrome *c* concentration of 2 $\times$ 10^{-8} mole per flask, the two determinations served as

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duplicates. In the case of heart and thigh muscles the maximal Q_{O_2} values were realized when the succinate was added from a Keilin cup at the completion of the equilibration period.

Results

The results obtained are summarized in Table I. It is apparent that significant changes in the succinoxidase content are observed only in the liver. In that tissue, supplementation of the diet with riboflavin results in a marked increase in the succinoxidase activity. This increase is most evident in those rats whose food intake is restricted to that of the basal group. Similar results, *i.e.* the marked influence of food restriction upon the restoration of enzyme content following vitamin therapy, have been previously noted (4, 5).

The data presented indicate clearly that one or more components of the succinoxidase system of rat liver are affected by the dietary intake of riboflavin. Such results are offered only as indirect evidence in support of the thesis that succinic dehydrogenase is a flavoprotein. The final proof that riboflavin enters into the structure of any of the components of the succinoxidase system must await the isolation in pure state of such components.

SUMMARY

1. The effect of a riboflavin deficiency in the rat upon the succinoxidase content of various tissues was studied.

2. The dietary intake of riboflavin was found to have a definite effect upon the succinoxidase content of liver tissue.

3. The results obtained were taken as *prima facie* evidence that one or more components of the succinoxidase system are flavoproteins.

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THE ISOLATION OF PROTOPORPHYRIN IX FROM FECES OF NORMAL AND ANEMIC RATS*

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(Received for publication, September 24, 1941)

Considerable evidence has accumulated in recent years indicating that urinary and fecal excretion of porphyrins may increase during recovery from anemic conditions of varying etiology. Dobriner and Rhoads (1) have discussed this subject in a recent review on porphyrins. It appears that a failure of porphyrin synthesis has not been recognized as a cause of impaired hemoglobin formation. Several observations are recorded in the literature, however, which made it desirable to study this possibility with rats suffering from nutritional anemia of iron deficiency and particularly of copper deficiency. Porphyrin compounds other than hemoglobin are affected by copper deficiency in rats. The activity of catalase (2), an iron-containing protoporphyrin IX compound (3), is decreased in the liver and kidneys of anemic, copper-deficient rats. Similarly the intensity of the spectral bands of cytochrome *a* (4) and the activity of cytochrome *c* oxidase (5) are reduced in some tissues of copper-deficient rats. Metalloporphyrins are generally considered to be involved in the activity of both cytochrome *c* oxidase and cytochrome *a*. Thus it appeared possible that copper might be concerned with the formation of a structural unit common to these compounds; namely, the porphyrin or heme nucleus. In addition, Watson and Clarke (6) and others (7, 8) have shown that reticulocytes

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contain larger amounts of protoporphyrin than erythrocytes; and a reticulocyte response and remission of copper-deficient rats is observed only following adequate copper therapy (9). These considerations prompted a study of porphyrin excretion by rats under conditions of iron and copper deficiency.

EXPERIMENTAL

The rats used in this work were kept on a milk diet, supplemented with manganese and either iron or copper or both (5). They were housed in cages with raised screen bottoms. The feces were collected twice each day and transferred to wide mouthed brown bottles containing glacial acetic acid. Preliminary observations indicated the presence of considerable amounts of porphyrin in the feces thus collected. Sufficient material was therefore accumulated to permit isolation and identification of the principal porphyrin present in the feces. The starting material for the isolation experiments usually represented the fecal yield from about 1000 rat days. The method of isolation of a crystalline porphyrin ester followed essentially the established procedure outlined by Dobriner (10) with minor modifications.

The ether extract of the feces containing the total porphyrins was washed with 1 per cent sodium acetate and then with water. The porphyrins were extracted from the ether with 5 per cent HCl and again taken up with ether after neutralization. This transfer was repeated twice. Without preliminary saponification the ether solution was extracted with 0.2 per cent HCl until the porphyrins of the coproporphyrin type were removed. Extraction of the ether with 5 per cent HCl removed all remaining porphyrins. After repeated washing of the HCl extract with petroleum ether the protoporphyrin was extracted from the HCl solution with several portions of chloroform. The chloroform solution was diluted with 2 volumes of ether and the porphyrin extracted with 5 per cent HCl. The porphyrin was precipitated from solution by neutralization with sodium hydroxide and sodium acetate. The precipitate was collected on the centrifuge (addition of about 1 ml. of chloroform facilitates this step; the chloroform is then evaporated from the precipitate). The porphyrin was dried with a little methyl alcohol and esterified by refluxing for 30 minutes with methyl alcohol containing 2 per cent dry HCl.

The methyl ester was precipitated by neutralization with dilute ammonia, collected on the centrifuge, washed with water, and dried with methyl alcohol. Crystallization was brought about by gradual addition of methyl alcohol to a concentrated warm chloroform solution of the methyl esther. After recrystallization from the same solvents and drying *in vacuo* at 60° the crystals melted at 221–223° (uncorrected).

Crystalline protoporphyrin dimethyl ester was obtained with this procedure from the feces of copper-deficient, iron-deficient, and normal rats on a milk diet. The yield of crystalline material varied from about 20 to 40 mg. per 1000 rat days.

The normal rats (fed Fe + Cu + Mn) were later transferred to a diet consisting of 18 per cent commercial casein, 68 per cent sucrose, 4 per cent salt mixture,[1] 10 per cent Crisco, and 80 γ of thiamine per 100 gm. of ration. In addition the rats received daily an aqueous solution of 0.5 gm. of liver extract (Abbott). The liver extract must have been almost free from porphyrin because a 5 per cent HCl extract of the acetic acid-ether extract from 100 gm. of this liver powder failed to show a fluorescence under ultraviolet light. Collection of feces from these animals was not started until 4 weeks after the change of the diet. Crystalline protoporphyrin dimethyl ester was again isolated from the feces of these rats. To obtain a crystalline product, however, it was necessary to saponify the crude porphyrins with 20 per cent NaOH (10).

Identification of Isolated Product—The solubility and the melting point of the isolated material indicated its identity with protoporphyrin IX dimethyl ester. This was substantiated by the mixed melting point with protoporphyrin IX dimethyl ester prepared from blood hemin. The most satisfactory method for the latter preparation appears to be that of Fischer and Pützer (11) in which pyridine hemochromogen is reduced with hydrazine and decomposed with concentrated HCl.

Reduction of protoporphyrin IX to mesoporphyrin IX is sometimes used for the purpose of identification. This reaction appears to involve large losses of porphyrin unless special precautions are taken. The following procedure was found to be quite satis-

[1] Kline, O. L., Bird, H. R., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, 11, 515 (1936).

factory: to 10 mg. of protoporphyrin dimethyl ester are added 100 mg. of ascorbic acid and 2 ml. of glacial acetic acid; 0.3 ml. of HI (sp. gr. 1.94, prepared conveniently from iodine and tetralin (12)) is then added and the mixture is boiled for 45 seconds. After cooling rapidly the solution is transferred into 50 ml. of ether and extracted twice with 15 ml. of an aqueous solution of 10 per cent sodium acetate and 3 per cent sodium sulfite. The ether solution is then washed with water and the mesoporphyrin is extracted with 5 per cent HCl. The extract is neutralized with sodium hydroxide and sodium acetate and the porphyrin is extracted with

TABLE I

Identification of Protoporphyrin IX

	M. p.	M. p. (mixed)		
	°C.	°C.		
Protoporphyrin methyl ester from hemin	221–223	221		
" " " " feces	221–223			
Mesoporphyrin " " " hemin	214	214		
" " " " feces	214			
	Absorbtion spectra; position of maxima in dioxane			
	$m\mu$	$m\mu$	$m\mu$	$m\mu$
Protoporphyrin methyl ester from hemin	630	575	538	504
" " " " feces	630	575	538	504
" IX dimethyl ester (13)	630	575	537	503
Mesoporphyrin methyl ester from hemin	620	565	528	495
" " " " feces	620	567	529	495
" IX dimethyl ester (14)	620	567	528	496

ether containing acetic acid. From the ether solution the porphyrin is extracted with 2.5 per cent HCl. The HCl solution is then washed with a mixture of 2 parts of ether and 1 part of chloroform. The porphyrin is precipitated from the acid solution with NaOH and collected on the centrifuge. After being washed with water it is esterified and the ester is separated and crystallized as in the case of protoporphyrin dimethyl ester. The yield by this procedure is 35 to 40 per cent. It could probably be improved by modifying the conditions of the reduction.

Table I summarizes the data which permit the conclusion that the porphyrin isolated was protoporphyrin IX. The absorbtion

spectra agree with those reported by Stern and Wenderlein (13, 14). Aside from protoporphyrin IX the feces also contained small amounts of other porphyrins. It was always possible to separate from the crude mixture of porphyrins a small fraction that was soluble in 0.2 per cent HCl; in one case there was isolated from this fraction 0.7 mg. of a crystalline methyl ester, m.p. 238–240°, probably coproporphyrin I. Coproporphyrin III also appeared to be present but it was not isolated. The crude porphyrins included a small fraction which was not extracted from ether by 0.2 per cent HCl or from 5 per cent HCl by chloroform. These fractions were not further investigated, however.

The possibility of urinary excretion of porphyrins was also considered. Urine from copper-deficient rats was collected in the following manner: The cages were underlaid with filter paper to soak up the urine. After removal of the feces and contaminated spots the filter paper was stored in glacial acetic acid. Periodically the acid was filtered with suction and used again for extraction and storage of the collections. Thus the urine from 1000 rat days was concentrated. After dilution and extraction with ether, neither a 5 per cent HCl extract of the ether solution nor the ether showed any fluorescence in ultraviolet light. The collected urine evidently did not contain more than traces of porphyrin.

DISCUSSION

The presence of porphyrin in the feces of rats has not been studied extensively. Hughes and Latner (15) observed relatively large amounts of porphyrin. Lemberg *et al.* (16) state in a footnote that "rats' feces contained a remarkably large amount of porphyrins, which consisted of protoporphyrin (predominant), of coproporphyrin and of deuteroporphyrin." Rimington and Hemmings (17) isolated the methyl esters of coproporphyrins I and III and the methyl ester of mesoporphyrin IX (after reduction of the protoporphyrin fraction) from the feces of rats on a diet containing 5 per cent of yeast. From the urine of the same animals they isolated the methyl ester of coproporphyrin III. The daily total porphyrin excretion of their rats was about 4 γ in the urine and about 80 γ in the feces. Thomas (18) reported daily excretion of about 100 γ of total porphyrin in the urine and feces of rats on a diet of milk and bread. However, he did not

characterize the type of porphyrin excreted. Under the conditions of the experiments recorded here protoporphyrin IX was the predominant porphyrin in the feces. The isolation of as much as 43 mg. of crystalline protoporphyrin dimethyl ester from the feces representing 1000 rat days indicates that the daily excretion was of about the same magnitude as that recorded by Rimington and Hemmings and by Thomas.

In this study the purification and isolation in pure form of the predominant fecal porphyrin were stressed. Such a procedure is naturally not quantitative. Conclusions regarding the amounts of protoporphyrin present in the feces of the rats on different diets would therefore be hazardous. A study of the quantitative aspects of this problem, particularly under different conditions of hematopoietic activity, is highly desirable and should be feasible in view of the relatively large amounts of porphyrin present in the feces.

The origin of the isolated porphyrin is not clear, but several possibilities must be considered: (*a*) exogenous origin, (*b*) endogenous origin, and (*c*) synthesis by microorganisms in the fecal material before collection and storage. No decision can be made on the basis of available information, however. Kämmerer and Gürsching (19) appear to have made the only observations on the presence of porphyrin in cow's milk. While the quantitative relations are not clear from their data, they observed weak lines of the fluorescent spectrum of coproporphyrin in two samples of raw milk. The intensity of the lines indicated the presence of not more than 1 γ of coproporphyrin in their samples. The fluorescent spectrum of protoporphyrin was not observed. It appears quite certain, however, that the amount of porphyrin excreted by the rats used in this study far exceeded the amount that was present in the diet.

Undoubtedly the rat can achieve the synthesis of protoporphyrin in the hematopoietic organs as well as in Harder's glands (see (18)). Under conditions of accelerated hematopoiesis the hemoglobin formed corresponds to a synthesis of about 2 mg. of protoporphyrin daily. In the normal course of events some protoporphyrin may find its way into the intestine. If such is the rce of the fecal protoporphyrin, it appears that even the verely anemic, copper-deficient rat can achieve the synthesis of

protoporphyrin. On the other hand, the possibility of bacterial synthesis of protoporphyrin in the intestine must be seriously considered. Kämmerer (20) first observed the formation of protoporphyrin by the synergistic action of bacteria on blood. More recently Jakob (21) demonstrated the presence of protoporphyrin in pure cultures of intestinal bacteria on media containing blood. It must be noted, however, that the incubation period used by Jakob was from 15 to 30 days and that the amounts of protoporphyrin observed were too small to permit isolation of the pigment. He also noted the synthesis of coproporphyrins, especially of coproporphyrin III, by aerobic bacteria on media devoid of hematin compounds. Von Mallinckrodt-Haupt reported only the formation of coproporphyrin under similar conditions (22) (the original reference is not available). The occurrence of fecal protoporphyrin has frequently been reported in humans with intestinal hemorrhage or following the ingestion of blood (see (23)). In the animals used in these experiments there was at no time evidence of hemorrhage, and hemoglobin determinations were purposely omitted. It appears rather unlikely, therefore, that blood pigment was the source of the protoprophyrin isolated. Its synthesis by the intestinal flora from other materials is quite possible, however, and the question of the origin of the fecal protoporphyrin must be left open. The isolation of protoporphyrin IX from the feces of both normal and copper-deficient rats indicates that either the animal or the intestinal flora can synthesize this pigment on a régime very low in copper.

SUMMARY

Protoporphyrin IX has been isolated from the feces of anemic copper-deficient rats, anemic iron-deficient rats, and normal rats on a milk diet. It has also been isolated from the feces of normal rats on a solid diet.

Dr. C. J. Watson, University of Minnesota, has kindly confirmed the melting points and absorbtion spectra of samples of fecal protoporphyrin and mesoporphyrin sent to him. His interest in this study is gratefully acknowledged.

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THE USE OF RADIOACTIVE COPPER IN STUDIES ON NUTRITIONAL ANEMIA OF RATS*

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When copper is fed to an anemic, copper-deficient rat, its effects on the hematopoietic organs are quickly noticeable. Most striking is the immediate change of the cytochrome *c* oxidase of the bone marrow from a very low to a normal or even accentuated activity (1). This is followed by a rapid reticulocytosis and a more gradual increase of the erythrocyte count and of the hemoglobin content of the blood (2). The possibility of a local accumulation of copper in the bone marrow, as suggested by Sarata (3), has been investigated with pigs but the analytical results have indicated that this does not occur to an appreciable extent (4). The amount of copper accumulated in the liver and the spleen under these conditions is also not very large. In two different laboratories it has been observed that the retention of copper during a period of copper therapy is very low even in severely depleted rats (5, 6). In view of the small quantities of copper involved and the limited amount of tissue obtainable from rats—the animal of choice for studies on nutritional anemia—present chemical methods of analysis were clearly not suitable for further pursuit of this problem. The development of the tracer

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technique with radioactive elements opened a new approach. With this technique it should be possible to determine whether copper actually did enter those tissues in which the effects of copper therapy are so rapidly manifest.

Several radioactive isotopes of copper have been described (see (7)). Only one, $_{29}Cu^{64}$, with a half life of 12.8 hours has properties suitable for physiological studies. Its successful use is governed by the following factors. (1) Copper, when fed to rats in large doses, is toxic. The amount given must be kept near the physiological limits usually employed. (2) The experiments must be limited to short periods in view of the short half life of radiocopper. (3) The material used must have a high radioactivity but a low content of total copper. (4) Chemical methods must permit a rapid and quantitative isolation and concentration of copper contained in the tissues.

To satisfy the first and third criteria just enumerated it became evident that $_{29}Cu^{64}$ prepared from $_{28}Ni^{64}$ (8) would be most suitable. Dr. Du Bridge and Dr. Van Voorhis of the Department of Physics of The University of Rochester kindly agreed to undertake the necessary bombardments. Targets of nickel weighing 200 to 250 mg. were bombarded with 6.7 m.e.v. protons for periods of 8 to 12 hours in the Rochester cyclotron. The copper content of the nickel was about 0.1 per cent, so that the whole sample generally contained not more than 0.2 to 0.3 mg. of copper. The radioactivity of these samples usually was of the order of 0.5 millicurie when they were used for feeding. Our requirements were therefore met in every respect by these preparations.

EXPERIMENTAL

Extraction and Concentration of Copper—The formation of a copper complex of diphenylthiocarbazone (dithizone) (9) provided a basis for the isolation and concentration of radioactive copper prior to feeding or counting. This method is rapid and simple. It permits the quantitative extraction of copper from acid solutions into known, small volumes of carbon tetrachloride which can be used directly for measurement of the radioactivity. The nickel targets (received from Rochester about 14 hours after conclusion of the bombardments) were dissolved with the aid of

hydrochloric and nitric acids. The solution was evaporated, diluted, and repeatedly extracted with a CCl_4 solution of dithizone (0.1 mg. of dithizone per ml. of CCl_4) until the color of the reagent remained unchanged. The CCl_4 extract was evaporated to dryness and ashed with 0.2 ml. of concentrated H_2SO_4 and a few drops of H_2O_2. The ash solution was diluted with 0.8 ml. of water. The acid was neutralized (phenol red) with redistilled ammonia and, after cooling, the volume of the solution was made up to 2.3 to 2.5 ml. 1 ml. of this solution containing from 0.1 to 0.15 mg. of total Cu was fed to each of two rats; 0.1 ml. was diluted to an appropriate volume to serve as a standard for the radioactivity counts and for the determination of total copper by a photoelectric dithizone method.[1]

Table I

Recovery of Radioactive Copper after Wet Ashing

Each flask contained 3 ml. of a solution of radioactive copper, extracted with dithizone. To Flasks 3 and 4, 2 gm. of liver were added before ashing.

Flask No.	1	2	3	4
Counts before ashing	320	314	309	306
" after "	325	318	306	303

From most of the tissues with the exception of the carcass and the different parts of the intestinal tract the copper was isolated in a similar manner. The tissues were weighed, transferred to glass-stoppered digestion flasks of about 60 ml. capacity, and ashed with sulfuric and perchloric acids. After complete digestion the acid solution was diluted and neutralized to about pH 2, with patent blue V (10) as indicator. To this solution 3 ml. of dithizone solution in CCl_4 were added and the copper was extracted by mechanical shaking for 15 minutes. The CCl_4 layer was used directly for measurement of the radioactivity. When a sample of radioactive copper was carried through this procedure either with or without added tissue, recovery was quantitative, as shown in Table I.

By measuring the radioactivity of an aqueous solution before and after extraction with dithizone under different conditions the

[1] Kuiken, K. A., Miller, J. A., and Schultze, M. O., unpublished data.

validity of this method could be checked. Copper dithizonate is a dissociable complex. When the acidity of the aqueous phase with which it is in contact is greater than pH 2.0, the complex begins to dissociate unless an excess of dithizone is present. With an excess of dithizone (about 20-fold) quantitative extraction can be achieved at pH 0.4 (Table II). Since an excess of dithizone did not affect the counts, a solution of dithizone containing 0.1 mg. per ml. of CCl_4 was used (in a few cases 0.2 mg. per ml. of CCl_4).

The feces and two portions of the intestinal tract were also wet ashed. Because they contained considerable radioactivity, the ash solutions of these samples were diluted with water to a

TABLE II

Effect of pH and Dithizone Concentration on Efficiency of Extraction of Copper

All vessels contained 6 ml. of radioactive Cu.

	7.2 γ dithizone per ml. CCl_4				100 γ dithizone per ml. CCl_4			
pH	0.20	0.80	1.00	1.50	0.40	0.60	0.91	1.40
Counts in 1.1 ml. aqueous phase								
Before extraction	1357	1462	1357	1462	290	290	290	290
After extraction	832	397	92	13	290	288	292	290
% extracted	38.7	73.9	93.2	99.1	100	100	100	100

definite volume and used directly for counting. The same applies to the carcasses which were dry ashed.

Counting of Radioactivity—The decay of $_{29}Cu^{64}$ yields positive and negative electrons of sufficient energy to permit their detection in a thin walled dipping counter (11). The instrument used was arranged so that the liquid in a cup could be raised over the counter. Samples with a relatively strong radioactivity were counted for a period of 4 minutes, weaker samples for longer periods. The accuracy of the counts varied with the radioactivity of the samples. The background counts were reproducible with a probable error of ±5 per cent. In stronger samples the accuracy was greater. Thus the mean probable error of the samples illustrated in Fig. 1 was ±0.8 per cent for Feces 654 and 2.5 per cent for Feces 464. Between counts of samples with considerable activity a background count was interposed to insure complete

removal of the previous sample from the instrument. In addition, the radioactive copper standard was counted at intervals to check the decay curve of the sample and thus to assure its freedom from other radioactive isotopes. The decay curve of radioactive material recovered from the feces of several rats to which it was fed was also determined. The slope of the curve corresponds to a half life of 12.8 hours (Fig. 1).

When counts are made in solutions of different electron densities, corrections for self-absorption by the solvent may be necessary. In the aqueous solutions used here no such corrections were found to be necessary (*i.e.*, within the limits of the counting accuracy)

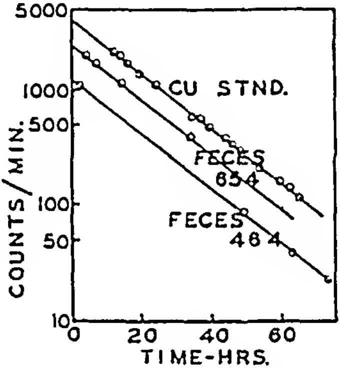

Fig. 1. Decay curves of radioactive copper. The count is plotted on a logarithmic scale. The slope of the lines corresponds to a half life of 12.8 hours, and is superimposed satisfactorily on the experimental points.

regardless of the salt concentration. In carbon tetrachloride solutions of copper dithizonate only 85 per cent of the corresponding activity in aqueous solution was registered in the counter. All counts made in carbon tetrachloride were therefore multiplied by 100/85. In view of the rapid decay of $_{29}Cu^{64}$, the results were calculated as the number of counts per minute at a given time of reference and the recoveries from the tissues were expressed in per cent of the number of counts fed.

Animal Technique—Young rats were made deficient in iron or copper as usual on a milk diet (1). They were used for these experiments when a severe anemia developed (hemoglobin about 3 gm. per 100 ml. of blood). Other observations have been

reported previously on rats which were in every respect comparable to these as far as we know. For this reason the radioactive material was fed with a small amount of milk in preference to administration by stomach tube. The rats were fasted for 14 to 16 hours before feeding to insure rapid consumption of the radioactive material. Afterwards milk was given *ad libitum.* During the therapeutic period the animals were kept in metabolism cages over large funnels. Because of the anticipated high radioactivity of the feces, special care was taken to collect and separate them from the urine quantitatively. Even so, slight contamination of the urine with fecal material may not always have been avoided. The rats were killed after 24 or 48 hours of copper therapy. The blood was collected in oxalate, measured, centrifuged, and the cells were washed twice with isotonic salt solution. Plasma and cells were analyzed separately. The gastrointestinal tract was removed and the stomach, the small intestine, and the cecum plus large intestine were analyzed separately, including their contents. Bone marrow was obtained as indicated previously (1). The liver, kidneys, spleen, and any other tissues removed were weighed and analyzed separately. The remainder of the body, referred to as the carcass, was dried for 4 hours, then ashed in a muffle furnace at a temperature not exceeding 500°.

DISCUSSION

A summary of the results obtained is presented in Table III. The relatively small amount of copper retained is again strikingly illustrated by all of the animals. The magnitude of this retention by copper-deficient rats is of the same order as that observed previously over a period of 7 days (6). In iron-deficient rats (which have been fed copper at all times) it is even lower. This difference in absorption and retention between deficient and plethoric rats recalls the observations of Hahn *et al.* (12) and of Austoni and Greenberg (13) on the absorption of iron. As with iron the absorption of copper and its retention by the tissues appear to be governed in part by the amount of iron present in the tissues. The results obtained with different tissues merit brief comment.

Blood—The per cent of copper recovered in the blood is calculated on the assumption that the blood volume is 7 per cent of

the body weight. Radioactivity was found in both plasma and blood cells. Of the radiocopper present in the whole blood the following was contributed by the plasma: in iron-deficient rats after 24 hours therapy, 68 per cent; after 48 hours, 61 per cent; in copper-deficient rats after 24 hours therapy, 75 per cent; after 48 hours, 49 per cent.

It appears that the erythrocytes are permeable to copper and that during the second 48 hours there is a continued increase of copper in the erythrocytes. The actual amount of copper accumulated in the blood during the periods studied is small, between 10 and 20 γ per 100 ml. of blood. Since no analyses are

Table III

Distribution of Copper Fed to Anemic Rats

The results are expressed in per cent of the amount of copper fed (100 to 150 mg. per rat).

Type of deficiency Duration of therapy No. of rats	Copper 24 hrs 9	Iron 24 hrs 4	Copper 48 hrs 10	Iron 48 hrs 4
	per cent	*per cent*	*per cent*	*per cent*
Blood	0.54	0.16	0.41	0.16
Liver	1.57	0.40	1.83	0.34
Kidneys	0.56	0.32	0.51	0.13
Spleen	0.14	0.02	0.12	0.02
Bone marrow	0.05	0.01	0.03	0.01
Carcass	2.54	1.43	3.30	1.89
Gastrointestinal tract + excreta	70.00	74.93	72.53	83.89
Retained	5.10	2.34	6.20	2.55
Recovered	75.40	77.27	78.73	86.44

available on the total copper content of the blood of comparable rats, the present results cannot be compared with the observations made with pigs (4).

Liver, Kidneys, and Spleen—Of the organs analyzed separately, the liver showed the greatest absolute retention of radioactive copper. Somewhat surprising was the appreciable localization of copper in the kidneys. In fact, per unit weight the kidneys contained more radioactive copper than any other organ analyzed. The relative copper retentions per gm. of tissue stand in the ratios of 1:0.5:0.3 for kidney, liver, and spleen, respectively, of rats on 24 hour copper therapy. In the same tissues of rats

on 48 hour copper therapy, the ratios are 1:0.7:0.3. Similarly in the iron-deficient rats the kidneys contained 2 to 3 times as much radioactive copper per gm. of tissue as the liver and spleen. Residual blood in these organs cannot be responsible for this relationship, because the blood in all cases contained less copper per gm. than the tissues. In iron-deficient rats Austoni and Greenberg found the highest specific accumulation of iron in the bone marrow, 24 hours after administration of the iron (13).

Bone Marrow—It is not possible to collect the bone marrow of rats quantitatively. From many observations we know that the bone marrow thus collected has a dry weight of about 35 mg. On this basis it is possible to compare roughly the entrance of

TABLE IV

Distribution of Copper in Gastrointestinal Tract and Excreta 48 Hours after Feeding 100 to 150 Mg. of Cu

The results are expressed in per cent of the amount of copper fed.

	10 copper-deficient rats	6 iron-deficient rats
	per cent	*per cent*
Stomach	2.7	5.3
Small intestine	3.2	4.4
Cecum + large intestine	16.9	16.3
24 hr. feces	25.0	26.0
48 " "	22.7	27.9
24 " urine	1.6	0.86
48 " "	0.43	0.53

therapeutic copper into the bone marrow and into other organs. If in the copper-deficient rats after 24 hours of copper therapy 0.05 per cent of the copper fed was present in 35 mg. of dry marrow, this corresponds to about 1.5 per cent per gm. In the liver (75 per cent moisture) of the same animals the amount of copper retained is about 1.4 per cent per gm. of dry weight. The deposition of copper 24 hours following copper therapy appears therefore to be about of the same order of magnitude in the bone marrow and the liver. Although the amount of copper entering the bone marrow in 24 hours is less than 0.1 γ, it is sufficient to elicit a great increase in cytochrome *c* oxidase activity and to initiate hematopoietic activity (1).

Gastrointestinal Tract and Excreta—As shown in Table IV most

of the copper fed was recovered from the gastrointestinal tract and the excreta. More detailed analyses of this fraction were made with the rats on 48 hour experiments (Table IV). It is evident that the elimination of copper is quite rapid under the conditions of our experiments. A small fraction appears to be excreted through the kidneys, even in severely deficient rats. We do not wish to stress the accuracy of the figures for urinary excretion of copper because the danger of contamination with fecal material of high radioactivity must not be underestimated. The figures given should be regarded as maximum values. With respect to urinary excretion, copper behaves much like manganese (14) but unlike cobalt (15).

The values given in Table III for the total recovery of administered copper appear quite low and we cannot offer a wholly satisfactory explanation. From three rats to which the copper was administered by stomach tube the average total recovery was 100.5 per cent (the figures from these animals have not been included in any of the tables). It appears therefore that the loss of radioactive copper did not occur in the analytical procedures used (see also Table I). The copper not consumed by the rats was also determined by analysis of any residue left in the feed dishes. In most cases this was a very small fraction, the average of twenty-three analyses being 0.14 per cent. When it was larger, the results were corrected for it. It appears most likely that the loss was mechanical, some of the material being deposited by the animals on the cages.

The experiments reported here confirm the earlier observations that only small amounts of copper are absorbed and retained by anemic rats. Although the retention by copper-deficient rats is greater than that by iron-deficient rats, it is still far below that observed when iron is administered to iron-deficient rats (13). The entrance of copper into the hematopoietic centers in amounts too small to permit detection by present chemical methods has, however, been demonstrated. The idea of the dependence of cytochrome *c* oxidase and hematopoietic activity of the bone marrow on the presence of copper is therefore fully reported.

SUMMARY

1. The radioactive isotope $_{29}Cu^{64}$ is suitable for biological experiments.

2. Copper-deficient rats retained more of a single therapeutic dose of copper than did iron-deficient rats. In both types of animals only a small fraction of the copper fed was retained.

3. The kidney, the liver, and the bone marrow show the highest relative retention of copper in 24 to 48 hours.

4. The entrance of therapeutic copper into the bone marrow of copper-deficient rats has been demonstrated.

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THE EFFECT OF THIOL COMPOUNDS ON THE ACTIVITY OF LACTOGENIC HORMONE*

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Recent studies of the effect of thiol compounds on various pituitary and related hormones have led to interesting results (1–4). When a method was described (1, 4) to free growth hormone of lactogenic, thyrotropic, and gonadotropic hormone contamination by the action of such reagents, preliminary studies of the unusual effect of thiols on the lactogenic hormone were reported. A more detailed study has now been carried out which has yielded results in general agreement with our first interpretation.

Investigations of the effect of thiol compounds on gonadotropins (2, 3) have shown the inactivating reaction to proceed at so slow a rate that appreciable inactivation was observed under the standard conditions only above a certain protein concentration. Less concentrated solutions of gonadotropins could be inactivated if the reaction were permitted to proceed at higher temperature, or for longer periods of time. When lactogenic hormone was treated with thiol compounds, the concentration of both reactants was found to be of importance, not only in regard to the rate of the reaction, but also for the final result. Besides this, the nature of the thiol reagent proved to be another important factor. Both cysteine and thioglycolic acid transform the protein into a very insoluble state and thereby may lead to an inactivation of

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† D. L. Meamber cooperated in the earlier part of this investigation.

hormone solutions, which is due only to the insolubility of the treated protein. This phenomenon will be referred to as "apparent inactivation." Besides this, treatment of the protein with a very great excess of cysteine or much less of thioglycolic acid leads to "true inactivation" of the hormone, with or without formation of a precipitate. Thus, by varying the experimental conditions, one may produce either true inactivation without precipitation of the protein, or apparent inactivation through precipitation of the active protein, or precipitation of the inactivated protein, or, finally, no change in activity or physical state of the hormone.

General Experimental Conditions

Treatment with thiol compounds was always carried out in solutions buffered with phosphates at pH 7.8; when 6 to 8 M urea was used as a solvent, it also was made up in this buffer. Cysteine hydrochloride was used, neutralized by two-thirds the amount of sodium carbonate. Thioglycolic acid was generally neutralized with 5 N sodium hydroxide; sodium chloride was added to obtain solute concentrations with this reagent similar to those with cysteine.

Experiments with Cysteine

Solutions containing 0.02 per cent or less hormone together with a 40-fold amount of cysteine (hydrochloride) showed no appreciable loss of hormonal activity, even when kept for 2 weeks at room temperature or for 48 hours at 37° (Table I; Table II, A, Experiments 1, 2). On the other hand, solutions containing higher concentrations of either or both reactants showed varying degrees of inactivation, accompanied by precipitation of the protein. In a series of experiments the ratio of cysteine to protein was kept constant (40:1), while the protein concentration was varied from 0.0075 to 0.2 per cent (Table I). It was observed that both the rate at which the turbidity and precipitation occurred and the amount of the precipitate increased with increasing protein concentration. The maximum of 90 per cent precipitation was reached rapidly with a hormonal concentration of 0.1 per cent. When these solutions or suspensions of lactogenic hormone treated with cysteine at various protein concentrations were assayed for

their biological activity, this was found to be proportional to the amount of unprecipitated protein remaining in solution. Thus, as previously stated, solutions of no more than 0.02 per cent

TABLE I

Treatment of Lactogenic Hormone with 40-Fold Amount of Cysteine; Effect of Hormone Concentration

Concentration of lactogenic hormone	Observations during reaction*	Protein in solution	Fraction injected	Assays in squabs†	
				Total dose	Crop reaction
per cent		*per cent*		*mg.*	
0.0075‡	Clear solution	100	Solution	0.2	+, +, +
0.0113	" "	100	"	0.15	2+, 2+, +
0.0225	Turbid solution; no ppt. forms	100	"	0.3	+, +, +
0.0225	" "	100	"	0.9	2+, 2+, +
0.035‡	Ppt. forms within 12 hrs.	55	Supernatant	0.4	3+, +, −
0.045	Ppt. forms within 1 hr.	Not determined	Suspension	1.8	2+, 2+, +
0.05	Ppt. forms within 15 min.	33	Supernatant	0.33	+, +
0.1	Ppt. forms within 5 min.	10	"	0.2	−, −, −
0.1	" "	Not determined	Suspension	4.0	−, −
0.2	Ppt. forms within 1 min.	11	Supernatant	0.7	−, −
0.2	" "	10	"	0.65	−, −
0.2‡	" "	12	"	1.0	−, −, +

* Reaction mixtures buffered to pH 7.8 were allowed to stand for 2 days at 18–22° before assay.

† In the 1 month-old Silver King squabs here used a + reaction may be obtained with approximately 2.5 to 5 I.U., a 2 + reaction with 5 to 10 I.U., and a 3 + reaction with over 10 I.U.

‡ The minimal effective dose of the hormone used for these experiments was 0.2 mg. per squab on intramuscular injection (0.1 mg. per squab on subcutaneous injection); the minimal effective dose of the starting material for all other experiments on this table was 0.25 mg. per squab. All injections in this investigation were performed intramuscularly.

hormone and 0.8 per cent cysteine remained clear and fully as active as the starting material, while protein concentrations of 0.02 to 0.05 per cent led to partial precipitation and inactivation.

TABLE II

Treatment of Lactogenic Hormone with Cysteine; Effects of Various Factors*

Experiment No.	Concentration: Lactogenic hormone	Concentration: Cysteine	Solvent, experimental conditions,† and observations	Assay in squabs: Total dose	Assay in squabs: Crop reaction
			A. Effect of temperature, time, solutes		
	per cent	*per cent*		*mg.*	
1	0.010	0.4	2 days at 37°, solution remains clear	0.4	3+, +, +, −
	0.015	0.6	40 hrs. at 38°, solution remains clear	0.23	+, +, +
2	0.01	0.4	10 days at 18–22°, solution remains clear	0.4	3+, 3+
3	0.02	0.8	Glycine added (to 7.2%); ppt. within 1 hr.; suspension	0.8	−, −
	0.02		Same, without cysteine	0.2	+, +, −
			B. Effect of changes in relative cysteine concentration		
1	0.02	8.0	Ppt. within 1 hr.; suspension	0.8	−, −, −
	0.01	4.0	9 days; ppt. within 12 hrs.; redissolved by addition of urea	0.36	−, −
	0.005	2.0	10 days, solution remains clear	0.4	−, −
2	0.01	2.0	10 " " " "	0.4	±, ±
3	0.1	0.4	Ppt. within 1 day; suspension	1.0	−, −
			C. Effect of 6 to 8 M urea as solvent		
1	0.2	8.0	Solution remains clear	0.4	3+, 2+, 2+
				0.3	+, +, +
				0.25	+, +, −
	0.04	1.6	" " "	0.4	3+, 2+
2	0.04	16.0	" " "	0.4	−, −
3	0.33	13.3	" " "	0.4	2+, 2+, −
			D. Effect of oxygenation‡ of reaction mixtures		
1	0.33	13.3	Aliquot of C-3 (urea as solvent)	0.4	2+, 2+, 2+
2	0.015	0.6	" " A-1 (phosphate buffer, no urea)	0.2	+, +

* All preparations used for these and all further experiments had a minimal effective dose of 0.2 mg. on intramuscular injection.

† Unless otherwise stated, reaction mixtures, buffered to pH 7.8, were allowed to stand for 2 days at 18–22° before assay.

‡ Oxygenated for 12 to 24 hours; nitroprusside test negative.

The small and constant amount of protein, however, which remained in solution at all concentrations of or above 0.1 per cent proved inactive at the levels tested.

It has been stated that a 40-fold amount of cysteine causes precipitation and inactivation only in solutions containing more than 0.02 per cent of the hormone. When glycine was added, however, together with this amount of cysteine, precipitation and inactivation occurred when the protein concentration was only 0.02 per cent, while glycine alone had no effect (Table II, A, Experiment 3). This could also be achieved by increasing the cysteine-protein ratio; thus a 400-fold amount of cysteine caused precipitation at a protein concentration of only 0.01 per cent (Table II, B, Experiment 1). On the other hand, smaller relative amounts of cysteine are required for the onset of precipitation at higher hormone concentrations; *e.g.*, 0.1 per cent (Table II, B, Experiment 3).

When cysteine treatment was performed in 6 to 8 M urea, no precipitate formed even at high protein concentrations (0.2 per cent) and no inactivation was noted when a 40-fold amount of cysteine was used (Table II, C, Experiments 1, 3). This is all the more surprising, since it has been shown that growth hormone and gonadotropins are more readily inactivated by cysteine in urea than in aqueous solutions (3, 4).

Since these experiments indicated that the cysteine-treated hormone was inactive only on account of its insolubility, it seemed important to dissolve the precipitated hormone and test the biological activity of the solution. Many experiments have been performed with this object with the following outcome. The precipitate is almost insoluble in water and was generally washed repeatedly before further use; it is very sparingly soluble (0.002 per cent) in phosphate buffer at pH 7.8 in which the original hormone is easily soluble. The precipitate is soluble at pH 8.5 to 9.0, *i.e.* in approximately 0.05 N sodium hydroxide, as well as in 6 to 8 M urea. When it was redissolved in urea without special precautions, it was found to have little if any activity (Table III, Experiment 1). If it was redissolved with exclusion of air, *i.e.* under nitrogen or hydrogen, or in the presence of cysteine, its activity was approximately that of the original hormone (Table III, Experiments 2, 3; Table IV, Experiment 3). When inactivated solutions, prepared by dissolving the precipitate

in urea without observing these precautions, were again treated with cysteine, reactivation of varying degree was achieved (Table III, Experiments 4, 5). When the precipitates were redissolved in weak alkali, less clear cut results were obtained (Table III,

TABLE III

Effect on Hormonal Activity of Conditions Employed for Dissolving Lactogenic Hormone Precipitated during Treatment with 40-Fold Amount of Cysteine

Experiment No.	Treatment of ppt.	Assays in squabs	
		Total dose	Crop reaction
		mg.	
1	Dissolved in 40% urea	1.6	−, −
		0.5	−, −
2	" " 40% " containing cysteine	0.5	2+, 2+
		0.25	2+, +, +
2a	Then oxygenated (12 hrs.)	1.6	−, −
3	Dissolved in 40% urea saturated with hydrogen	0.5	2+, +
		0.25	+, +, +, +, −
4	Dissolved in 40% urea, stirred 1.5 hrs., then treated with cysteine	1.0	2+, 2+, 2+
4a	Then oxygenated (12 hrs.)	1.0	−, −, −
5	Dissolved in 40% urea, stirred 6 hrs.	2.0	+, −
5a	Then treated with cysteine	0.9	3+, +
		0.3	−, −
6	Washed thoroughly, dissolved in 0.05 N NaOH (—SH test positive)	1.4	3+, 2+
		0.4	2+, +
6a	Same	0.4	+, +
6b	Unwashed ppt. dissolved in 0.05 N NaOH + trace of cysteine	0.5	3+, 2+
	Same after thorough washing, under nitrogen (—SH test positive)	1.0	2+
6c	Washed ppt. (—SH test positive) dissolved in 0.05 N NaOH + trace of cysteine	0.4	−, −, −
6d	Washed ppt. (—SH test negative) dissolved in 0.05 N NaOH, under nitrogen	0.36	+, +, −
	Same aerated	0.36	+, +, +

Experiment 6); sometimes activity was recovered and sometimes not.[1] It appeared that much handling, such as repeated

[1] It will be shown in the following paper that the number of reducing groups in the thiol-treated protein was found to be lower and more irregular when it was redissolved in alkali than upon direct determination.

washing, might lead at times to inactivation. Solutions of thoroughly washed protein were not regularly inactivated by aeration.

Besides its insolubility, the sensitivity to autoxidation clearly differentiates the cysteine-treated precipitated form of lactogenic hormone from the untreated hormone. Dilute and therefore not precipitating solutions of the hormone and cysteine were consequently exposed to oxygen after 2 days standing; similar mixtures at higher concentrations but in urea and therefore not precipitated were treated similarly. Both types of solution were oxygenated longer than necessary for the oxidation of all the cysteine (negative nitroprusside test) and then assayed for biological activity. The fact that both types of solutions were found fully active (Table II, D) is interpreted as evidence that under conditions of cysteine treatment which do not lead to precipitation most if not all of the hormone remains unchanged.

While it has been shown that a 40-fold amount or less of cysteine caused only an apparent hormone inactivation, a very much greater excess of this amino acid (200- to 400-fold) led to true inactivation. This could be shown either in very dilute protein solution (0.005 per cent) or at higher concentrations in 8 M urea, conditions which prevent precipitation of the protein; it could also be demonstrated for the precipitated protein, redissolved in the presence of cysteine (Table II, B, Experiments 1, 2; C, Experiment 2).

Experiments with Thioglycolic Acid

Thioglycolic acid caused precipitation of 90 per cent of the protein above certain protein concentrations, as does an equivalent amount of cysteine. (In one experiment of this type the rate of precipitation was followed by nitrogen analyses. The concentration of protein was 0.11 per cent, and of thioglycolic acid 2.5 per cent. Of the nitrogen, 41 per cent was found in solution after 13 minutes, 16 per cent after 44 minutes, and 12 per cent after 2 days. The suspension was then assayed at a high level in squabs (6.2 mg.) and found inactive.) However, while a 200- to 400-fold amount of cysteine was needed to produce true hormone inactivation, thioglycolic acid produced this effect at one-fiftieth of the molar equivalent of that amount (Table IV). As with cysteine, this true inactivation is independent of protein con-

centration; when dilute hormone solutions (0.01 per cent) were treated with a 23-fold amount of thioglycolic acid (equivalent

TABLE IV

Comparison of Effect of Cysteine and Thioglycolic Acid on Lactogenic Hormone

Experiment No.	Concentration		Conditions† and observations during reaction	Assays in squabs‡	
	Hormone	Thiol reagent*		Total dose	Crop reaction
	per cent	*mM per ml.*		*mg.*	
1	0.01	0.025§ Cy.	10 days at 18–22°, solutions remain clear	0.4	3+, 3+
	0.01	0.025 Th.		0.4	−, −
				0.8 (Dialyzed)	−, −
2	0.01	0.025 Cy.	40 hrs. at 37°, solutions remain clear	0.4	3+, +, +, −
	0.01	0.025 Th.		0.8	−, −
	0.01	0.005 "		0.4	2+, 2+, +, −
3	0.2	0.5§ Cy.	Ppt. forms after a few min.; redissolved by addition of urea before assay	0.8	4+, 3+
	0.2	0.5 Th.		0.8	−, −
4	0.13	0.33 Th.	Ppt. appears after 10 min.	0.8	±, −
	0.13	0.066 "	Ppt. appears after 20 min. (both redissolved as above)	0.8	2+, 3+
5	0.33	0.83§ Cy.	In 8 M urea, solutions remain clear	0.4	2+, 2+, +
	0.33	0.45 Th.		0.8	−, −, −
6	0.1	0.625 Cy.	In 8 M urea, solutions remain clear	0.4	2+, 2+, +, +
	0.1	0.05 Th.		0.4	−, −
	0.1	0.025 "		0.4	±, ±, ±, ±
	0.1	0.0125 "		0.4	±, +

* Cy., cysteine; Th., thioglycolic acid.

† 2 days at 18–22°, in pH 7.8 phosphate buffer, unless otherwise stated.

‡ Intramuscular injection (0.15 to 0.2 mg. of pure lactogenic hormone gives + reaction under such conditions).

§ This represents a cysteine-protein ratio of 40:1.

to a 40-fold amount of cysteine), no precipitate formed but the solutions were inactive (Table IV, Experiments 1, 2). When the same experiment was performed at a 10-fold or higher hormone

concentration, nine-tenths of the protein precipitated, inactive even when redissolved with those precautions[2] which regularly permit recovery of hormonal activity from precipitates produced by treatment with an equivalent amount of cysteine (Table IV, Experiments 3, 4). Also treatment with thioglycolic acid in 6.7 M urea solution leads to inactivation of the hormone without formation of a precipitate (Table IV, Experiments 5, 6). From these experiments it appears that thioglycolic acid is about 50 times more effective than cysteine in inactivating lactogenic hormone.

DISCUSSION

When lactogenic hormone is treated with a 40-fold amount of cysteine, formation of a precipitate is observed above a certain protein concentration; loss of biological activity is found to parallel this precipitation. No inactivation is apparent when cysteine treatment is performed in more dilute solution or in 6 to 8 M urea solution, conditions which do not lead to the formation of a precipitate. When the precipitate is redissolved under conditions which prevent autoxidation, its potency resembles that of the untreated hormone. All these findings indicate that the action of a 40-fold amount of cysteine on lactogenic hormone does not cause true inactivation of the hormone. It causes its transformation into an insoluble state. It will be shown in the following paper that this insoluble protein differs from the untreated hormone in containing many reducing groups and in particular thiol groups formed by reduction of disulfide bonds. The susceptibility of the precipitated hormone to autoxidation mentioned above is easily explained by this fact. Apparently reoxidation of the reduced protein does not regenerate the original hormone in active form.[3]

The conditions necessary for the precipitation of the hormone seem to merit a more detailed discussion. It has been noted

[2] The simplest and most reliable method of redissolving the hormone in active form is by addition of an equal amount of urea to the precipitated protein-thiol reaction mixture. The precipitate dissolves in this highly concentrated urea solution and can then be diluted for assay.

[3] Attempts of various authors to achieve regeneration of the activity of other proteins have not been successful (5, 6), with the possible exception of partly reduced insulin (7).

that the degree of precipitation is determined entirely by the concentrations of protein, cysteine, and other solutes. It has also been mentioned that the solubility of the precipitated form in phosphate buffer at pH 7.8 (0.002 per cent) is considerably lower than the hormone concentration necessary for beginning precipitation from reaction mixtures (0.02 per cent); also that lactogenic hormone treated with cysteine in dilute solution or in urea is not as sensitive to oxidation as the precipitated form. The simplest interpretation of all these facts seems to be the following. The reaction between hormone and a 40-fold amount of cysteine rapidly[4] reaches an equilibrium when only a small fraction, possibly about 10 per cent of the hormone, has been transformed into the insoluble form. If the original hormone concentration is below 0.02 per cent, this small fraction remains in solution and no changes are noted. The same is true when urea is used as solvent, even at higher protein concentrations, owing to the solubility of the reduced form under these conditions. Any factor, however, which raises the concentration or decreases the solubility of the insoluble form leads to precipitation, thus shifting the equilibrium of the reaction, until almost all the hormone is transformed into the insoluble state. This can be effected by an increase in either protein concentration (with proportionally increased cysteine) or in solute concentration, or in the cysteine-protein ratio.

If the cysteine-protein ratio is increased to more than 200, the action of the cysteine changes in character; true inactivation ensues, *i.e.* inactivation which is not due to either insolubility or reoxidation of the reduced hormone. This true inactivation produced by a very great excess of cysteine can well explain the previously reported fact that treatment of the hormone with a 40-fold amount of cysteine, besides causing the precipitation of 90 per cent of the protein, also leads to inactivation of the 10 per cent which remains in solution. It is obvious that with precipitation of most of the protein the cysteine-protein ratio in solution is greatly increased.

When thioglycolic acid is used instead of cysteine, true inactivation occurs at much lower relative thiol concentrations. This

[4] At 18–22° the appearance of turbidity varied from 1 minute to 12 hours, according to the concentrations employed.

thiol compound is apparently about 50 times more effective than cysteine in inactivating lactogenic hormone, while similar concentrations of the two reagents are needed for precipitation of the protein.[5] This difference is surprising in view of the fact that the oxidation-reduction potential of the two thiols has been shown to be the same (8).

SUMMARY

1. Treatment of lactogenic hormone with more than a 200-fold amount of cysteine causes true inactivation. Less cysteine (40-fold) may lead to an inactivation of hormone solutions of sufficient concentration by causing the transformation of the hormone into a very insoluble state. If this insoluble protein is redissolved under conditions which prevent autoxidation, it is fully as active as the untreated hormone. If formation of a precipitate is avoided by performing the cysteine treatment in very dilute protein solutions or in 6 to 8 M urea, no inactivation occurs.

2. Thioglycolic acid is approximately 50 times more effective than cysteine in causing true inactivation of lactogenic hormone, although similar amounts of the two thiols are needed to cause precipitation of the protein.

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[5] The protein precipitates obtained by treatment with equivalent amounts of the two thiol reagents differ in various respects. Their biological difference has been pointed out; their chemical properties will be discussed in the following paper.

THE EFFECT OF THIOLS ON THE REDUCING GROUPS OF LACTOGENIC HORMONE*

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Various proteins are known to contain reducing groups. The strongest reducing groups in proteins appear to be thiol groups, while weaker reducing properties are attributed to tyrosine and tryptophane (1). A number of biologically active proteins are affected by treatment with reducing agents, such as mercaptans; the specific activity of some is enhanced, while others are inactivated. It is generally believed that the action of these compounds is confined to the reduction of protein disulfide bonds but no conclusive evidence for this has been brought forth.[1]

In the preceding paper, the action of cysteine and thioglycolic acid on the lactogenic hormone of the anterior pituitary has been described. An attempt to explain the findings there reported has led to this investigation of the nature of the reducing groups in the lactogenic hormone before and after treatment with thiol compounds.

Methods and Materials

A number of different reagents were used under varying conditions to permit estimation and differentiation of reducing and reducible groups in the protein and specifically cysteine and

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[1] Stern and White (2) have used phosphotungstate in determining the reducing groups of insulin treated with thioglycolic acid; it has become apparent during the present investigation that other than thiol groups in proteins may reduce this reagent.

cystine in hydrolysates. To avoid autoxidation, hydrolyses were performed in a current of oxygen-free nitrogen.

Phosphotungstate—This reagent was used for estimation of reducing groups (presumably thiol) in unhydrolyzed proteins (2) as well as in hydrolysates. In either case the reaction proceeded at pH 6.7 in 5 M urea (reagents as for Mirsky and Anson's modification (3) of the Folin-Marenzi method (4)); the color was read on a Cenco photelometer 10 minutes after addition of the reagent. In the case of the unhydrolyzed protein, a precipitate formed after addition of the reagent and was centrifuged off before the reading was taken. The color intensity was compared with that of standard cysteine solutions, plotted on a curve (which was a straight line on semilogarithm paper over a wide range of concentration), and expressed in terms of this amino acid. In the absence of groups reducing phosphotungstate, treatment of the protein or its hydrolysate with sulfurous acid and subsequent use of the reagent as described above yielded colors which were regarded as most probably due to cystine or protein disulfide bonds; they were read on standard cystine curves and expressed in terms of this amino acid.

Sullivan Reaction (*5*)—This method was used for determination of cysteine and in its absence of cystine in protein hydrolysates. Also with this reaction standard curves were constructed and found reliable, with close observation of the conditions specified by the author, particularly in regard to freshness of the reagents. To determine the limits within which this method was applicable to the determination of cysteine in the presence of cystine, control experiments with casein hydrolysates or known mixtures of these two amino acids or both were performed. If the cystine to cysteine ratio in such mixtures was 9 or above, cysteine values were obtained which were too high by 2 to 3 per cent of the cystine present. (Cystine in the absence of cysteine gives apparent cysteine values of 3 to 8 per cent: the lower value at higher cystine concentration; *e.g.*, 3 mg. of cystine yield 0.091 mg. of apparent cysteine; 0.9 mg. of cystine, 0.046 mg. of cysteine; 0.3 mg. of cystine, 0.023 mg. of cysteine.) This interference by cystine disappeared rapidly with increasing relative amounts of cysteine and, with a ratio of cystine to cysteine of 5, correct values are obtained even for as little as 0.08 mg. of cysteine.

Nitroprusside and Ferricyanide—The nitroprusside test was used only as a qualitative indication of the presence of reducing and presumably thiol groups. It was used as an indicator in experiments in which proteins were titrated in 6.8 M urea with 0.001 M potassium ferricyanide (9). Ferricyanide was also used by Anson.

Reducing groups in the protein were also estimated by its ability to reduce cystine in 0.5 N KOH (3). The cysteine thus formed was determined with phosphotungstate, as described above.

The reaction of thiol groups with iodoacetamide was used in an indirect manner as an indication of the number of such groups formed from disulfide groups by reduction of the protein. For this purpose the reduced protein was treated at pH 8.0 with an excess of iodoacetamide[2] for 2 hours at room temperature, dialyzed, hydrolyzed, and the cystine content of the hydrolysate, determined by the Sullivan method, compared with that of the untreated protein. If it is assumed that R—S—CH_2—COOH does not yield thiols under the conditions of hydrolysis or reduction, the cystine found can be derived only from the unreduced protein disulfides.[3]

The preparations of lactogenic hormone[4] used in this investigation contained 25 to 30 I.U. per mg. Several of the preparations had appeared homogeneous when used in electrophoretic and solubility studies (6). The hormone solutions were precipitated

[2] Iodoacetamide was prepared according to Anson's procedure and recrystallized to constant melting point. With iodoacetic acid, less rigorously purified, great difficulties and apparent loss of cystine were encountered.

[3] Carboxymethylcysteine and its amide were used in control experiments (NH_2—CH(COOH)—CH_2—S—CH_2—CO—R; R = OH or NH_2). Samples of the acid were kindly put at my disposal by Professor H. B. Lewis and by Dr. H. Tarver; the amide was prepared from cysteine and iodoacetamide. These compounds did not yield color when the Sullivan method for cystine was used. They did cause a slowly and progressively increasing color when treated with phosphotungstate and sulfite. Since, in the absence of disulfides, phosphotungstate is slowly reduced by sulfite directly, it appears probable that carboxymethylcysteine catalyzes this reaction. The Folin method can therefore not be used for the determination of cystine in the presence of such compounds.

[4] For some of the lactogenic hormone preparations I am indebted to Dr. W. R. Lyons and Dr. C. H. Li.

by thorough dialysis, and then dried over phosphorus pentoxide *in vacuo*. Preparations of the reduced protein were obtained by treating solutions containing at least 0.1 per cent of the protein, as described in the previous paper, with a 23-fold amount of thioglycolic acid or an equivalent amount (40-fold) of cysteine at pH 7.8 for 2 days at room temperature. The precipitate which formed was then centrifuged off and freed from thiol reagent by repeated washing with distilled water, saturated with oxygen-free nitrogen. After four to five washings the nitroprusside test was generally negative in the supernatant but washing was always continued for two to three times more after this point had been reached.

Discussion of Results (Summarized in Table I)

Reducing Groups in Lactogenic Hormone—The nitroprusside and phosphotungstate tests are negative, even if the hormone is dissolved in such denaturing agents as 8 M urea or guanidine hydrochloride (7). Also the hydrolysate contains neither cysteine nor other groups reducing phosphotungstate. All this indicates that thiol groups do not exist in this hormone; this fact has been confirmed by different methods by Anson[5] and by Li and coworkers (6). When the protein was treated with cystine in 0.5 N KOH, it reduced this amino acid, but not at pH. 9. (10 mg. of hormone, acting on 52 mg. of cystine in 1 cc. of 0.5 N KOH at

[5] When these facts were brought to the attention of Dr. M. L. Anson, he agreed to perform a few experiments with this protein. He, too, arrived at the conclusion that —SH groups probably do not exist, but that reducing groups of unknown nature are present in this protein. Dr. Anson performed the following experiments: (*a*) Negative nitroprusside test in guanidine hydrochloride solution, even when the precautions are taken which have been described (8). (*b*) Positive nitroprusside test in guanidine hydrochloride solution if before the addition of nitroprusside the protein is exposed to strong cyanide in alkaline solution. Cyanide reduces —S—S— to —SH. (*c*) No reduction of ferricyanide in neutral Duponol-PC solution as described (9). (*d*) 5 mg. of hormone, 1 cc. of 0.1 M sodium borate buffer, pH 9.2, 0.5 cc. of 0.1 M ferricyanide, 10 minutes at 37°; 1 cc. of 0.0009 M ferrocyanide formed. Exposure to borate alone does not produce —SH, as shown by negative nitroprusside test. No ferrocyanide formed if the hormone is exposed to ferricyanide for 30 minutes in phosphate buffer (pH 7.4). Besides these experiments the author is indebted to Dr. Anson for valuable suggestions regarding this paper and concerning further work to be done.

*Content of Cystine, Cysteine, and Other Reducing Groups in Lactogenic Hormone before and after Treatment with Cysteine and Thioglycolic Acid**

Method		Lactogenic hormone untreated	Cysteine-treated hormone	Thioglycolic acid-treated hormone
		per cent	*per cent*	*per cent*
Sullivan (5)	Cystine	3.0 (4 determinations)		
	Cysteine	None (<0.4)	0.0 (2 determinations)	1.5 (1.4, 1.5, 1.6)
Phosphotungstate In Hydrolysate (3)	Disulfides	3.0 ± 0.1 (6 determinations)		
	Reducing groups	None (<0.2)	1.4 (1.3, 1.4, 1.5)†	3.1 ± 1.5 (8 determinations)‡
Direct (2)	Disulfides	2.2 ± 0.3 (4 determinations)		
	Reducing groups	None	1.4 ± 0.2 (7 determinations)	3.2 ± 0.3 (5 determinations)
Ferricyanide (6)	Reducing groups in 5 M urea, 10 min., 37°	(pH 9.2) 2.2§ (" 7.4) None§	(pH 6.7) *ca.* 1.6	(pH 6.7) *ca.* 3.0
After treatment with iodoacetamide	Cystine (Sullivan)	2.9 (2.8, 3.0)	1.5 (1.7, 1.3)	0.74 (0.72, 0.75)
Sulfur		1.79 ± 0.03‖ (5 determinations)		2.12 ± 0.05 (5 determinations)

* All values for disulfides and reducing groups are determined and expressed in terms of cystine and cysteine respectively, while it is recognized that only the Sullivan method is specific for these amino acids.

† When the precipitates were dissolved with sodium hydroxide before hydrolysis, 0.68 ± 0.08 per cent cysteine was found in six determinations and only traces in two experiments. It appears possible that these lower values may be due to a catalytic action of OH^- ions on the autoxidation of the reduced protein.

‡ Great and unexplained variations were encountered in eight experiments in which the Folin method was used with hydrolysates of thioglycolic acid-treated hormone; no similar difficulties were met with in other methods or protein fractions.

§ We are indebted to Dr. M. L. Anson for these values (*cf.* foot-note 3).

room temperature, caused the formation within a few hours of 0.2 to 0.36 mg. of cysteine, the yield increasing with time; this could be determined directly with phosphotungstate, without removal of the protein, since the latter does not reduce the reagent.) It is therefore concluded that lactogenic hormone does not contain thiol groups but does contain groups of unknown nature which are more easily dehydrogenated than any non-thiol groups heretofore described in proteins.[5]

Disulfide Groups in Lactogenic Hormone—The cystine content (assuming no cysteine) of hydrolysates of the hormone was found to be 3.0 per cent in four determinations by the Sullivan method. Six determinations by the Folin method also indicated a cystine content of 3.0 per cent. The good agreement between the values obtained by these two methods indicates that the hormone contains no disulfides other than cystine. Four estimations of these groups in the unhydrolyzed protein indicated 2.2 per cent; this lower value is believed to be due to the fact that not all disulfide bonds in the protein are equally accessible to the reducing action of the sulfite ion.

Reducing Groups in Thiol-treated Lactogenic Hormone—Thiol-treated hormone precipitates give positive tests for reducing groups with all methods employed, either when redissolved in 6 to 8 M urea or in alkali or upon hydrolysis. As was reported in the preceding paper, thioglycolic acid causes true inactivation of the hormone at very much lower concentrations than does cysteine, both causing the transformation of the protein into an insoluble form at approximately equivalent concentrations. Similarly the number of reducing groups was found to differ between protein precipitates obtained by treatment with equivalent amounts of these two thiol reagents. With all five methods of analysis employed, hormone preparations treated with thioglycolic acid were found to contain from 67 to 130 per cent more reducing groups than the cysteine-treated hormone.

The reducing groups of the thiol-treated protein could be (1) protein thiol groups, formed by reduction of disulfides, (2) reducing groups other than thiol, formed from unknown protein groups, and (3) thiol groups due to the reagent's having been absorbed by or combined with the protein.

A number of different analytical methods were therefore used

in an attempt to elucidate the nature of the reducing groups of the thiol-treated lactogenic hormone. From the findings it appears certain that protein disulfide bonds have been reduced and thiol groups formed; it also becomes evident that other reducing groups of unknown nature have appeared; the third possibility, the combination of a small amount of the thiol reagent with the protein, also appears probable, at least in the case of thioglycolic acid. The basis for these conclusions will now be discussed.

1. The finding of appreciable amounts of cysteine by the Sullivan method (1.5 per cent) in the protein treated with thioglycolic acid is regarded as unequivocal proof for the reduction of protein cystine radicals. This is confirmed by the results of determinations of unchanged cystine after treatment of the reduced proteins with iodoacetamide. From a quantitative point of view cysteine determinations indicate the reduction of 30 and 50 per cent of the total cystine by cysteine and thioglycolic acid respectively, while determination of the residual protein cystine indicates the reduction of 50 and 75 per cent respectively.

2. The existence of reducing groups other than thiol in the protein after treatment with cysteine and thioglycolic acid is indicated by the fact that consistently more phosphotungstate is reduced by the treated proteins than can be accounted for by their cysteine content (Sullivan); this is so when the proteins are treated directly with the Folin reagent as well as in the case of the hydrolysates. Since aliquots of the same hydrolysate were repeatedly found to contain about 50 per cent more "cysteine" by the Folin than by the Sullivan method, the difference cannot be due to losses of cysteine through autoxidation during hydrolysis. As previously stated, the good agreement of the cystine values for the untreated hormone obtained by these two methods appears to exclude the existence of other disulfides in the hormone. The only remaining interpretation appears to be the existence in the reduced protein of groups other than thiol which reduce phosphotungstate. A few experiments with ferricyanide indicate that this reagent is reduced in neutral urea solution to a similar extent that phosphotungstate is by the reduced proteins. The number of reducing groups determined by these reagents, directly or in hydrolysates, corresponds to 47 and 105 per cent of the total cystine, after cysteine and thioglycolic acid treatment respectively.

3. The possibility that some of the thiol reagent had combined with the protein in such a manner that it was not removed by repeated washing was tested by comparison of the sulfur content of the treated and untreated proteins. Three samples of protein treated with thioglycolic acid, one of which had been dissolved and dialyzed for a long time after the usual washing, contained 2.1 per cent of sulfur, which is 0.3 per cent higher than the sulfur content of the untreated protein. This would indicate a stable combination of approximately 1 per cent thioglycolic acid with the protein; if it is assumed that the thiol groups of these thioglycolic acid molecules are still free in the protein addition compound, their reducing power might account for about 1.3 per cent "cysteine," as determined by phosphotungstate or ferricyanide. This value would then have to be subtracted from the 3.0 to 3.2 per cent "cysteine" determined by these reagents, leaving reducing groups, corresponding to about 2 per cent of the protein when calculated as cysteine. These thioglycolic acid molecules could not account for or contribute to the cysteine determined by the Sullivan method. A similar addition of cysteine to the protein is possible when this thiol is used for reduction. This factor again could not explain the discrepancy between total reducing groups and cysteine in the treated hormone. It thus follows that stable combination of thiol compounds with proteins is a possible occurrence. While this has to be kept in mind in analyzing data concerning the reducing power of such proteins, it is not established that the thiol groups of the reagent will be free in such compounds. Even if it is assumed that in the case under consideration they are free, this does not affect the validity of the first two findings; *i.e.*, the reduction by thiol compounds of protein disulfide bonds and of other unknown groups in lactogenic hormone.

The author wishes to express his gratitude to Professor Herbert M. Evans for helpful advice and criticism received during this work.

SUMMARY

1. The presence of 3.0 per cent cystine in lactogenic hormone has been demonstrated. No cysteine was found in hydrolysates nor did the unhydrolyzed protein reduce the usual thiol reagents. It did reduce cystine in strongly alkaline solution.

2. Treatment of lactogenic hormone with thiol compounds causes (*a*) the reduction of disulfide bonds to cysteine thiol groups, (*b*) the appearance of groups of unknown nature which reduce phosphotungstate and ferricyanide in neutral solution, and (*c*) the probable formation of stable addition compounds between the protein and the thiol compound.

3. Thioglycolic acid was found to cause about twice as much reduction as an equivalent amount of cysteine.

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THE RESPONSE OF LIPID METABOLISM TO ALTERATIONS IN NUTRITIONAL STATE

II. THE EFFECTS OF OVERNUTRITION ON THE POSTABSORPTIVE LEVELS OF THE BLOOD LIPIDS OF THE DOG*

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The desirability of providing data on the maximum fluctuations that can occur in the lipid levels of the blood and liver under a variety of experimental conditions was pointed out in a previous communication from this laboratory (1). The response of the blood lipids to fasting and chronic undernutrition has already been recorded (1). The present investigation deals with the changes in cholesterol, phospholipid, and total fatty acids in the blood of dogs subjected to an excessive caloric intake that resulted in a condition of *obesity*, in which the animals gained as much as 80 per cent of their initial body weight in a period of 90 days.

According to Hurxthal (2), the cholesterol content of the blood is normal in obese subjects. Others, however, have reported that obesity does influence the lipid levels of the blood. Thus Mayer *et al.* (3) stuffed geese and found that after some time a high blood fat content was present. Rony and Levy (4) state that the average fasting level of total fatty acids in obese patients was somewhat higher than in normal patients, whereas cholesterol contents were practically the same. Solotoreva *et al.* (5) also reported a hyperlipemia with considerably increased lecithin and cholesterol levels in obese subjects.

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EXPERIMENTAL

Twelve adult dogs were used in the present study. Before the caloric intake was increased, each animal received a diet adequate in calories, proteins, salts, and vitamins. They were fed twice daily (at 8.00 a.m. and 4.00 p.m.) 30 gm. of lean meat and 6 gm. of sucrose per kilo per day. Vitamins A and D were supplied as cod liver oil and the vitamin B complex as galen B (vitab, Type II liquid[1]). Each animal received once daily 2 gm. of Cowgill's salt mixture (6) along with the meat and sucrose fed at 4.00 p.m. 2 gm. of bone ash were also furnished with each dietary mixture.

Following this control period, three experimental procedures were adopted: (1) Overnutrition was induced by doubling the caloric intake of the control period; (2) overnutrition was induced by feeding raw pancreas; (3) marked obesity was induced and then followed by fasting. During the entire period of observation the daily intake of salts, cod liver oil, and rice bran concentrate was kept constant in all animals recorded below. During overnutrition, food not eaten voluntarily was forcibly fed.

Whole blood was used for lipid analyses. From animals that were fed, blood was taken just before the morning meal. Thus at the time blood was sampled, all dogs were in a postabsorptive state, having ingested their last meal 16 hours before the removal of the blood. In the case of animals subjected to fasting, blood samples were removed between 8.00 and 9.00 a.m. Microoxidative procedures were employed for lipid determination. These have been described elsewhere (7). Free cholesterol was determined in an acetone solution from which the phospholipids had been precipitated.

Overfeeding with Mixed Diet of Meat and Sucrose

The blood lipid changes associated with weight gain produced by increasing the intake of meat and sucrose were investigated in eight dogs. The results obtained on two of these (Dogs N-57 and N-58) are recorded in Table I and are discussed below. In the six other dogs (Nos. N-15, N-16, N-25, N-29, N-32, and N-33) observations were extended over periods of 91 to 104 days; during

[1] This concentrate was generously supplied by The Vitab Corporation, Emeryville, California.

the first 14 to 27 days they received the control diet, whereas during the last 71 to 90 days they were subjected to overnutrition by doubling the intake of meat and sucrose. This treatment produced marked increases in weight. At the end of the periods of observation, the lowest gain in weight was 54 per cent above the initial body weight and the highest 83 per cent. Blood lipid determinations were made on at least two occasions during the control period and from four to eight times during the period of overnutrition. The blood lipid changes observed in these six dogs are not recorded in Table I but are discussed in the following paragraphs.

Cholesterol—In four of the dogs (Nos. N-25, N-29, N-32, and N-33) the levels of total cholesterol in the blood remained practically unchanged throughout the periods in which their weights were rapidly increasing. Thus the final values for total cholesterol were 192, 190, 241, and 196 mg. per 100 cc. of whole blood, while the *highest* corresponding control values were 194, 189, 220, and 189 mg., respectively. In these four dogs, the maximum values observed at other intervals after the intake of excessive calories had been instituted did not exceed the highest control values by more than 24 mg. In the remaining two dogs, increases of 52 and 75 mg. were associated with the gain in weight.

Phospholipids—An excessive intake of calories produced variable responses in the postabsorptive levels of the blood. In five dogs (Nos. N-15, N-16, N-25, N-29, and N-33) the final phospholipid values did not exceed their corresponding control values by more than 45 mg. Despite fluctuations, the maximum values observed during the periods of overfeeding were between 15 and 74 mg. above their corresponding highest control values. 71 days after Dog N-32 had been placed on a diet that led to a gain of 61 per cent in its body weight, the phospholipid content of its blood had increased by 87 mg. per 100 cc.

Total Fatty Acids—This lipid constituent responded similarly to a high caloric intake in four of the six dogs examined. In Dogs N-25, N-29, N-32, and N-33, the final blood values did not exceed the highest control value by more than 49 mg. In Dog N-15 the final value was 80 mg. per 100 cc. above the control, and in Dog N-16 an even greater rise, namely 110 mg. per 100 cc. of blood, was noted.

Overfeeding with Diet Containing Raw Pancreas

The blood lipid changes produced by overfeeding with raw pancreas are shown in Table I. In the four dogs (Nos. N-44, N-45, N-46, and N-36) investigated, observations were extended over a period of 41 to 59 days. During the control period, each animal received a diet of lean meat and sucrose, and the amounts fed were sufficient to maintain the initial weight of the dog. This was followed by overfeeding with raw pancreas for 28 to 32 days, during which time each animal received 125 to 150 gm. of the glandular tissue twice daily in addition to the control diet.

The total lipids remained practically unchanged despite the gain in weight that resulted from the ingestion of pancreas in addition to the control diet. The final values in Dog N-36 and in Dog N-46 were only slightly higher than the highest control values, whereas in Dog N-44 the level for total lipids observed 28 days after the feeding of raw pancreas was begun was below the control value. In Dog N-45, an increase of 84 mg. in total lipids was found at the end of the period of feeding with raw pancreas.

In no case did a significant rise in phospholipids, total cholesterol, or total fatty acids of the blood follow the ingestion of extra calories in the form of raw pancreas.

Changes Produced in Blood Lipids by Fasting Obese Dogs

In five dogs (Nos. N-44, N-45, N-46, N-57, and N-58) recorded in Table I, the blood lipids were compared during three nutritional states. (1) The normal nutritional state is referred to as *control.* This period of observation extended over 8 to 20 days, during which several blood samples were removed for analyses. (2) During the period labeled *overnutrition,* the dogs received a high caloric diet for 64 to 92 days. Pronounced gains in weight occurred in all dogs. At the end of these periods of observation Dogs N-44 and N-46 had gained 9.1 and 8.8 kilos, and their weights at this time were 76 and 74 per cent respectively above their control weights. (3) In the third nutritional state, referred to as *fasting,* all food except vitamin concentrates and salts was withheld.

Cholesterol—In three of the animals (Dogs N-44, N-45, and N-57) the highest values for total cholesterol observed during the period of overnutrition were in close agreement with the control values.

TABLE I

Effect of Nutritional State on Lipid Metabolism

All lipid values are expressed as mg. per 100 cc. of whole blood.

Dog No.	Treatment	Days on treatment	Diet per meal			Weight	Cholesterol			Total fatty acids	Phospholipid	Total lipid
			Meat	Sucrose	Raw pancreas		Total	Free	Ester			
			gm.	gm.	gm.	kg.						
N-57 ♀	Control	20	190	38		11.7	160	119	41	375	375	535
	Overnutrition	64	380	76		18.2	165	101	64	410	441	575
	Fast	3				17.5	137	100	37	358	284	495
	"	5				17.3	128	114	14	344	332	472
	"	7				16.4	121	93	28	364	334	485
	"	30				14.0	118	91	27	424	312	542
N-58 ♀	Control	12	140	28		9.4	141	114	27	335	374	476
	Overnutrition	70	280	56		14.0	170	110	60	427	475	597
	"	82	280	56		14.6	165	119	46	395	497	560
	Fast	5				13.8	152	105	47	385	433	537
	"	10				13.0	143	124	19	344	332	487
	"	30				11.3	130	103	27	372	358	502
N-44 ♂	Control	8	180	36		11.5	146			474	400	620
	"	12	180	36		12.0	150			474	425	624
	"	13	180	36		12.0	151			475	439	626
	Overnutrition	8	180	36	125	12.6	147			450	427	597
	"	28	180	36	125	14.3	143			410	428	553
	"	42	360	72		16.9	145	106	39	382	360	527
	"	60	360	72		18.8	168	118	50	510	441	678
	"	89	360	72		21.4	168	100	68	473	361	641
	"	92	360	72		21.1	160	106	54	417	389	577
	Fast	4				19.2	108	84	24	300	198	408
	"	15				17.2	104	94	10	335		439
	"	23				16.0	104	103	1	277	273	381
N-45 ♂	Control	12	200	40		13.6	195			369	377	564
	"	13	200	40		13.6	189			367	397	556
	Overnutrition	8	200	40	125	14.4	186			390	369	576
	"	28	200	40	125	15.6	193			455	423	648
	"	42	400	80		18.5	176	120	56	449	417	625
	"	89	400	80		23.2	181	103	78	467	354	648
	"	91	400	80		23.0	185	119	66	393	382	578
	Fast	4				21.8	130	108	22	340	260	470
	"	6				19.3	121	95	26	344	261	465
	"	15				18.5	119	83	36	285		404
	"	23				17.2	99	91	8	224	228	323

TABLE I—*Concluded*

Dog No.	Treatment	Days on treatment	Diet per meal: Meat	Diet per meal: Sucrose	Diet per meal: Raw pancreas	Weight	Cholesterol: Total	Cholesterol: Free	Cholesterol: Ester	Total fatty acids	Phospholipid	Total lipid
			gm.	gm.	gm.	kg.						
N-46 ♂	Control	8	180	36		11.9	134			363	338	497
	"	12	180	36		11.5	143			356	288	499
	"	13	180	36		11.5	164			360	343	524
	Overnutrition	8	180	36	125	12.1	174			428	380	602
	"	28	180	36	125	13.5	166			393	392	559
	"	42	360	72		15.9	165	109	56	404	370	569
	"	60	360	72		17.8	184	115	69	466	468	650
	"	79	360	72		19.8	174	98	76	498	418	672
	"	91	360	72		20.7	164	98	66	368	340	532
	Fast	4				19.3	124	85	39	316	260	440
	"	10				18.0	117	87	30	354	313	471
N-36 ♀	Control	14	200	40		13.3	165			360	337	525
	"	27	200	40		13.4	164			355	347	519
	Overnutrition	20	200	40	150	14.3	179			424	368	603
	"	32	200	40	150	15.2	187			410	377	597

In the other two dogs, increases of 29 and 50 mg. were noted. During fasting after the period of overnutrition, the levels of total cholesterol in all dogs decreased somewhat from the levels found at the end of overnutrition. The most striking fall occurred in Dog N-45, where the level fell from 185 to 99 mg. at the end of the fast. The average decrease in all dogs at the end of the fast was 32 per cent below the overnutrition level.

Total Fatty Acids—Overnutrition produced variable responses in the levels of total fatty acids. Although wide fluctuations occurred, increases were noted in most dogs. Thus, while the levels in Dogs N-57 and N-44 rose but slightly following the increased caloric intake, the values in the other three dogs varied as much as 92 to 135 mg. above the control level. The fatty acid response to fasting following overnutrition was also variable. Decreases in this constituent were quite pronounced in some dogs but not in others. In Dogs N-44 and N-45 a fall of 140 to 169 mg. respectively was observed following the fast, whereas only small changes were found in Dogs N-57, N-58, and N-46.

Phospholipids—The phospholipid levels also showed considerable fluctuation during the production of obesity. In Dogs N-57, N-44, and N-45 only slight changes occurred, but in Dogs N-58 and N-46 increases of 123 and 125 mg. respectively above control values were found. When obese dogs were fasted, pronounced decreases were observed in the phospholipid levels of them all. Differences of 157 to 243 mg. were noted between the levels during overnutrition and the levels during the period of fasting.

DISCUSSION

It was previously shown that acute fasting in which all dietary constituents with the exception of vitamins and salts were withheld failed to alter markedly the total cholesterol, total fatty acids, and phospholipids of whole blood (1). The present study shows that even while enormous amounts of fat are being deposited in the subcutaneous, abdominal, and such regions, a lipemia does not occur. In no instance was the level of any constituent higher than that normally found in the dog. Thus both observations suggest that wide variations in dietary régime need not markedly influence the blood lipids in the normal dog.

The observation that large amounts of raw pancreas fail to produce striking rises in the blood lipids of the normal dog is worthy of note, particularly in view of the previous work that showed that the feeding of raw pancreas has a profound effect upon the blood lipids of depancreatized dogs maintained with insulin (8). When raw pancreas is not included in the diet, the level of all blood lipids in the latter type of animal falls below normal, whereas the administration of the glandular tissue results in concentration of total cholesterol, phospholipids, and total fatty acids of the blood well above the normal. In some cases, values for total lipids well over 800 mg. and for total cholesterol over 250 mg. per 100 cc. of whole blood were observed 34 to 41 days after the ingestion of 125 gm. of raw pancreas twice daily in addition to a meat-sucrose diet. The stability of the lipid levels of *normal* dogs overfed with raw pancreas suggests that overnourishment is not the cause of increased blood lipids in pancreas-fed depancreatized dogs.

According to Bloor (9), whether or not an increase in blood fat occurs in fasting depends on the nutritional state of the dog. A dog that failed to show an increase in blood fat during fasting did

show this when stuffed with fat food for a week before fasting was induced. Terroine (10) observed both increases and decreases in fatty acids of the blood during fasting. This worker also ascribed the variation in response of fatty acids to differences in the nutritional states of the dog studied.

It was found in the present investigation, however, that *even in the presence of massive deposits of body fat fasting failed to raise the level of any of the lipid constituents in the blood.* On the contrary, decreases were found, and in several cases the drop was more pronounced in the obese dog than in the dog of normal nutritional state (1). At first sight this observation is somewhat surprising, for, as noted above, fasting itself as well as the production of obesity itself led to little or no change in the concentration of blood lipids. The explanation for this apparent paradox lies in the fact that, while obesity did not lead to a hyperlipemia, it nevertheless had a tendency to establish the blood lipids at the upper level of the normal range. It is the fall from these higher but still normal levels that gives to fasting its greater effect in the obese dog than in the dog of normal nutritional state.

SUMMARY

1. The experimental production of obesity (in which dogs were made to increase their weights by as much as 80 per cent) led to little or no rise in the total cholesterol content of the blood. There was a tendency for total fatty acids and phospholipids to rise in the obese dog, but this response was not uniform in the animals studied.

2. Raw pancreas (which readily influences the blood lipids of the completely depancreatized dog maintained with insulin) failed to produce significant changes in the blood lipid constituents in the *normal* dog.

3. Fasting appears to produce a more pronounced fall in the blood lipids in the obese dog than in the dog of normal nutritional state.

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DOES HYDROXYGLUTAMIC ACID OCCUR IN MILK PROTEINS?*

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The history of hydroxyglutamic acid[1] is a peculiar one. It was first announced by Dakin (1), who reported 10.5 per cent of it in casein. While this value has since been incorporated in many tables and text-books, Dakin (2) later found lower yields, down to 2 or 3 per cent. The characterization of the acid seemed at the time adequate, and the preparation from it of what was described as the "*p*-nitrophenylosazone of malic semialdehyde" appeared sufficiently definite.

3 years later, Jones and Johns (3) reported that they had found 10 per cent of it in lactalbumin, and Rimington (4) still later reported a considerable amount in the "phosphopeptone" he obtained from casein. Recently (5) he (and others) revised his analysis. The serine, threonine, and hydroxyglutamic acid which he originally reported have now become serine, isoleucine, and glutamic acid. We know of no better illustration of the inadequacy of the means which have, in the past, been available for the identification of hydroxyamino acids.

More recently isolations have been reported (6) which seemed "surprisingly easy" to Abderhalden (7, 8), even though he, with others, has recently claimed that he has isolated at least 3 per cent of hydroxyglutamic acid from casein.

But many workers who have in recent years desired to isolate hydroxyglutamic acid for experimental purposes have been unsuccessful or have obtained products which they believed to contain considerable amounts of the desired substance, but have not adequately characterized it. We have been privileged to

* Presented in substance before the American Society of Biological Chemists at Chicago, April, 1941 (*Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, 140, p. xcviii (1941)).

[1] Throughout this article β-hydroxyglutamic acid is meant.

analyze two or three such products, but with most discouraging results. Such negative results are seldom mentioned in the literature.

Still, Harington and Randall (9) reported their failure to isolate this acid, and Chibnall and Bailey[2] describe in some detail methods by which the "hydroxyglutamic acid fraction" of casein may be reduced almost to the vanishing point, while accounting for much of their material as glutamic and aspartic acids.

Of recent reports that hydroxyglutamic acid has been successfully isolated, we consider that of Gulland and Morris (10) the most nearly convincing. Their evidence cannot easily be set aside; but they claim only 0.3 per cent of hydroxyglutamic acid in casein.

For the evident confusion in this field, two explanations can be offered. Hydroxyglutamic acid is found in a residue which remains after all better known amino acids have been removed; such a situation is bound to be unsatisfactory. Furthermore, there have never been available suitable methods for the estimation, study, or characterization of hydroxyamino acids.

It is the purpose of this paper to demonstrate that the amount of hydroxyglutamic acid in the proteins of milk is very small (if it is present at all) and to specify tests which samples supposed to contain this acid should be required to meet.

We have shown elsewhere (11) that periodic acid reacts rapidly and quantitatively with β-hydroxy-α-amino acids. The reaction for serine is

$$\begin{array}{ccccccccccccc} OH & & NH_2 & & & & O & & & & O & & \\ | & & | & & & & \| & & & & \| & & \\ CH_2 & — & CH & — & CO_2H & \xrightarrow{HIO_4} & CH_2 & + & NH_3 & + & CH & — & CO_2H \end{array}$$

The corresponding reaction for hydroxyglutamic acid would be

$$\begin{array}{ccccccccccccccccc} CO_2H & & OH & & NH_2 & & & & CO_2H & & O & & & & O & & \\ | & & | & & | & & & & | & & \| & & & & \| & & \\ CH_2 & — & CH & — & CH & — & CO_2H & \xrightarrow{HIO_4} & CH_2 & — & CH & + & NH_3 & + & CH & — & CO_2H \end{array}$$

Van Slyke *et al.* (12) have reported that synthetic hydroxyglutamic acid,[3] reacts rapidly and quantitatively with periodic acid in potassium carbonate solution, as judged by the liberation

[2] Chibnall, A. C., and Bailey, K., American Chemical Society, Boston, September, 1939.

[3] See foot-note 4.

of ammonia. This is clearly a minimum test which any sample of hydroxyglutamic acid must meet. The isolation of a derivative of malonic acid semialdehyde would make the demonstration conclusive.

But there is another angle of appraisal which can also be applied. By the use of periodic acid we have developed satisfactory methods for the determination of threonine (13) and serine (14) in protein hydrolysates. With the same reaction, but by measuring ammonia liberation, it is possible to measure "total hydroxyamino acids;" and we have the statement of Van Slyke *et al.* (12) that this should include hydroxyglutamic acid, if present. We accordingly present the accompanying balance sheets for certain proteins.

It is necessary here to point out that the values reported for threonine and serine in our previous papers (13, 14) on the determination of these amino acids were designed to show reproducibility (which was our logical first interest), but they do not represent exact values for the proteins in question, since they were not corrected for moisture and ash. The *ratios* remain unaffected. The values reported in Table I have been corrected in the manner indicated in each case.

The molecular ratios of threonine to serine found in these proteins approximate closely to integers. The ratio for casein of 0.598 is clearly 3:5. For lactalbumin, the corresponding value (0.973) is 1:1, and for lactoglobulin we find even more precisely 4:3 (1.335). The values given seem to confirm the precision of our methods, and may also help to define these two minor proteins of milk.

It will be clear that if our results are accurate there is *no* hydroxyglutamic acid in the hydrolysates of any of the proteins here studied. But it has often been pointed out that the conditions of hydrolysis employed (refluxing for 24 hours with 20 per cent hydrochloric acid) may be too severe,[4] and may cause destruction

[4] Through the greatly appreciated cooperation of the Editors, we have received from one of them a small sample of synthetic β-hydroxyglutamic acid hydrochloride. As judged by ammonia evolution after treatment with periodate, the sample was of very good quality. With it, we carried out the following experiments. The hydrochloride (90 mg.) was added to 1.25 gm. of casein, and the mixture hydrolyzed by our usual method (refluxing for 24 hours with 20 per cent hydrochloric acid). The ammonia liberated by periodate was determined on an aliquot, and compared with that value obtained from a sample of casein run in parallel. The difference cor-

TABLE I

Balance Sheet for Hydroxyamino Acids in Casein, Lactalbumin, and Lactoglobulin

The results are expressed as per cent. The actual value for each protein is given in the first column, and the equivalent value, calculated as serine, in the second.

	Casein*		Lactalbumin†		Lactoglobulin‡	
Total hydroxyamino acids		9.05		9.27		7.10
Serine	5.82	5.82	4.74	4.74	3.12	3.12
Threonine	3.95	3.48	5.24	4.62	4.72	4.16
Other hydroxyamino acids, by difference		−0.25		−0.09		−0.18

* A good commercial casein from which the fat had been somewhat exhaustively extracted. Analysis showed it to contain 14.00 per cent N, and the reported analyses are *corrected* on the assumption that pure casein contains 15.80 per cent N.

† We are grateful to Dr. D. B. Jones for this preparation of lactalbumin. It was not particularly highly purified and is not the preparation on which Jones and Johns reported analyses. It contained 13.86 per cent N, and the reported values are *corrected* on the assumption that it should have contained 15.43 per cent N.

‡ The lactoglobulin used was a very pure (several times recrystallized) product kindly placed at our disposal by Dr. A. K. Balls. Unfortunately, we had only 0.3 gm., and the values reported probably deserve less credit than those listed above for casein and lactalbumin. The values given are calculated from the total N content of the solution, on the assumption that lactoglobulin contains 15.30 per cent N (value supplied by Dr. Balls and Dr. Axelrod).

responded to a recovery of 56 per cent of the original hydroxyglutamic acid. We also hydrolyzed casein with sulfuric acid according to the directions of Dakin (1), and determined the "total hydroxyamino acids" in the usual way, by estimating the ammonia liberated on treatment with periodate. The value obtained was indistinguishable from that obtained by our usual method of hydrolysis. We also used the same method of hydrolysis with sulfuric acid (1) on a mixture of hydroxyglutamic acid hydrochloride and casein. The recovery of hydroxyglutamic acid in this case was 70 per cent. The amount of destruction of authentic hydroxyglutamic acid in the experiments we quote does not vary greatly according to the method used. It is larger than we should have predicted, but it is by no means large enough to account for the apparently complete absence of hydroxyglutamic acid in our hydrolysates. We should have found half of it as readily as all of it. And we feel obliged to raise the point that the rate of destruction of this acid under various conditions is relevant only in case it was originally present.

of hydroxyglutamic acid. We can only answer that previous claims of destruction have presented evidence of only *partial* destruction, and in the entirely possible case that the hydroxyglutamic acid was not there, this question would not arise at all. But no amino acid determinations approach perfection too closely and it is in order to examine certain specific possibilities of error.

With regard to the threonine determinations, we have considered the objection that malonic acid semialdehyde, which should be produced by the action of periodic acid on any hydroxyglutamic acid present, is a β-aldehydo acid, and as such likely to be decarboxylated to give acetaldehyde. In that case, it would be counted as threonine.

We are inclined to disregard this factor, for a number of reasons.[5] (1) We do not believe that this reaction would take place at an important rate at room temperature and at the pH of bicarbonate. (2) Only a *rapid* action could be important, for the evolution of acetaldehyde from threonine approaches zero quite rapidly after an hour or less; any reaction of the sort suggested would have to proceed at such a rate as to be *complete* in an hour or less. (3) Rose has found (by feeding experiments) substantially the same value for threonine in casein. As threonine is an "essential" amino acid, this agreement is important.

With regard to formaldehyde formation, we admit at once that hydroxylysine, if present, would be indicated as serine. This would have no effect on the argument. But any sugar which happened to be present would be indicated, mole for mole, as serine; and in such a case each mole of formaldehyde formed from a carbohydrate would leave room for 1 mole of hydroxyglutamic acid.

We have attacked this aspect of the question in two ways. (1) We have acetylated the amino acids selectively in aqueous solu-

[5] The gift sample of hydroxyglutamic acid hydrochloride (see foot-note 4) has allowed us to confirm this view experimentally. The hydrochloride (24 mg.) was treated as for a threonine determination. The "acetaldehyde" evolved in terms of 0.02 N iodine solution was 0.03 cc. after 0.5 hour, and 0.12 cc. after 1.5 hours. Acetaldehyde from threonine comes over more rapidly. The persistency here suggests that acetaldehyde *is* formed from hydroxyglutamic acid by the reactions suggested, but at a negligible rate. Even after 1.5 hours, the amount recorded is only 2 per cent of that initially present. This reaction, then, clearly does not influence the conclusions put forward in this paper.

tion, without important acetylation of any sugars which might be present, and attempted to determine any sugars that survived the hydrolysis by the serine method (15).[6] The results are not as yet entirely clear cut, but they offer little hope for a significant decrease in the serine content of casein. (2) A further experiment, in which 5 per cent of lactose was added to casein before hydrolysis, led (by the methods referred to in the previous paragraph) to serine values low enough to indicate substantially complete destruction of the carbohydrate. The uncertainty introduced into our serine determinations by surviving carbohydrate would not be greater than the equivalent of 0.2 per cent of hydroxyglutamic acid in the protein.

We can only conclude that (*a*) we have never personally met *any* evidence for the existence of hydroxyglutamic acid in proteins; (*b*) we have sufficient faith in those chemists who have in the past reported it to conserve the hope that it exists in some degree in milk proteins; (*c*) we insist that the content surviving hydrolysis in casein and in lactalbumin cannot exceed the 0.3 per cent claimed by Gulland and Morris; (*d*) if the hope expressed above, that *some* of the acid exists, is fulfilled, our methods are by a small margin less accurate than we have believed them to be; (*e*) we shall in any case have supplied, for the first time, methods for the conclusive recognition of future samples which may be supposed to be hydroxyglutamic acid.

EXPERIMENTAL

Determination of Total Hydroxyamino Acids—The method used is modified from that of Van Slyke and Cullen (16) for the determination of ammonia from urea, and is briefly as follows:

Apparatus—Two pairs of 25 × 200 mm. test-tubes are connected in series as a gas train. The first tube of one pair is fitted with a separatory funnel, the stem of which reaches nearly to the bottom and serves as the gas inlet tube.

Procedure—The first tube of each pair (Tubes I and I′) contains an aliquot of the protein hydrolysate representing, in not more than 5 cc., 100 to 200 mg. of protein. In addition it contains 15 cc. of saturated K_2CO_3 solution and 2 drops of Nujol. The second

[6] We confirm our original finding (11) that acyl groups on nitrogen effectively inhibit the reaction of hydroxyamino compounds with periodic acid.

and fourth tubes (Nos. II and II′) contain 25 cc. each of standard 0.02 N HCl and 2 drops of methyl red indicator.

To start the reaction, an excess of periodic acid (1 to 2 cc. of 0.5 M acid)[7] is added to *one* of the reaction tubes (No. I or I′) through the funnel, and a current of air (washed through dilute sulfuric acid) is drawn through the solution for 1 hour at the rate of about 2 liters per minute. At the end of this time the NH_3 in the two receivers is separately estimated by titration with 0.02 N alkali. The *difference* between the two amounts of NH_3 found is considered to be due, mole for mole, to the decomposition of hydroxyamino acids, and is most conveniently calculated as "serine equivalent." 1 cc. of 0.02 N acid is equivalent to 2.10 mg. of serine. Sometimes a not quite insignificant additional amount of NH_3 may be recovered by aerating for an additional half hour.

Since NH_3 reacts readily with aldehydes, its smooth evolution requires that other amino groups be present to displace it from aldehyde combination. In the analysis of relatively pure hydroxyamino acid fractions, this would require the addition of several moles of a suitable non-volatile amine; we have used alanine. None of the protein hydrolysates tested required this addition. Even in the case of silk proteins the necessity for the addition of alanine was most doubtful.

Hydroxyproline does not yield any NH_3, and there is no indication that any other amino acid which does not contain an aliphatic hydroxyl group interferes.

As an alternative procedure, it is possible to determine the "amide NH_3" and "hydroxyamino acid NH_3" in the same sample. After the former has been obtained in the usual way, periodic acid is added, and the NH_3 thus formed determined separately. This procedure requires more time, but permits a certain economy of material when necessary.

Appraisal of Carbohydrate in Casein Hydrolysate[8]—The hydrolysate from 0.3125 gm. of casein was selectively acetylated under suitable conditions in aqueous solution with 2 moles (based on N content) of acetic anhydride. After treatment of aliquots with

[7] The periodic acid concentration given as "0.5 N" in reference (13), p. 95, line 25, should have been "0.5 M."

[8] The method is tentative as yet and will be discussed in detail elsewhere. The results given should, however, prove fairly typical.

periodic acid, acetaldehyde and formaldehyde were determined. The apparent threonine content was 0.11 per cent, or about 3 per cent of the value obtained before acetylation. It was assumed that this implied 97 per cent acetylation of the threonine present, and the same value was assumed for serine.

The formaldehyde gave 2.3 mg. of dimedon precipitate, of which 1.3 mg. should be formed from 3 per cent of the serine. The remaining 1.0 mg. is equivalent to 0.2 per cent glucose in the hydrolyzed casein.

In another experiment, 5 per cent of lactose was added to casein before hydrolysis (24 hours with 20 per cent HCl), and threonine and serine determined. The corrected values were 3.99 and 5.46 per cent respectively. This serine value is lower than the average previously found, and suggests no additional survival of carbohydrate from the added lactose.

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A DIRECT PHOTOELECTRIC COLORIMETRIC METHOD FOR THE DETERMINATION OF DIODRAST AND IODIDES IN BLOOD AND URINE*

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The organic iodine substances, diodrast and hippuran, were shown several years ago by Landis, Elsom, Bott, and Shiels (1) to have the highest clearances in the human kidney of any known substance. In 1938, Smith, Goldring, and Chasis (2) described methods for estimating the "effective blood [plasma] flow" (diodrast clearance), the plasma "filtration fraction" (the ratio of inulin clearance to diodrast clearance), and the functioning "tubular excretory mass" (diodrast T_m) of the kidney by determining the excretion of diodrast, at low and high plasma levels, and the simultaneous excretion of inulin. Application of these measurements in health and disease has led to new concepts of renal vascular dynamics.

In order to simplify the analytical technique of diodrast clearance and T_m measurements and, thereby, increase their usefulness for clinical studies, we have devised the method here described. This new procedure depends upon the conversion of diodrast to iodate, color development by liberation of iodine from the iodate, intensification of the yellow iodine color, and finally measurement of the color intensity in a photoelectric colorimeter. Our method, though very rapid, yields accurate results in the analysis of plasma or urine filtrates containing 1 γ or more of diodrast iodine per cc.

Principles of Method

Conversion of Diodrast to Iodate—The method is based on the fact that diodrast can be recovered quantitatively from protein-

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free filtrates of plasma or urine when cadmium is used as the precipitating agent (Table I).

Diodrast or iodide is readily oxidized to iodate by heating with bromine.[1]

Liberation of Iodine from Iodate—Excess bromine is removed before the liberation of elementary iodine by the addition of an alcoholic phenol solution. The resulting brom-phenols are colorless.

Color is developed by the addition of potassium iodide, according to the following equation.

$$KIO_3 + 5KI + 6H_3PO_4 = 3I_2 + 6KH_2PO_4 + 3H_2O$$

Intensification of Color—The yellow color of the free iodine is augmented by the addition of a large excess of potassium iodide, through the formation of a triiodide complex.[2]

$$I_2 + KI \rightleftarrows KI_3$$

This reaction greatly increases the sensitivity of the method. The intensified color bears a curvilinear relationship to the concentration of iodate (or diodrast iodine) in the solution (Fig. 1). Though the above reactions have long been known, we have been unable to find any previous application of this intensification in colorimetric microanalysis.

Measurement of Color Intensity—The No. 490 Evelyn filter, which Sendroy has demonstrated to be suitable for the analysis of iodate at higher concentrations (4), is not sensitive enough for the determination of plasma diodrast iodine at the low levels that must be maintained during diodrast clearance measurements. Under

[1] This observation has been made independently by Alpert (3), who has recently described a titrimetric method for the analysis of diodrast. In his method, which was published after the completion of our work, the iodate is measured by thiosulfate titration. This titration method, though considerably less rapid than our colorimetric procedure, should prove very useful in laboratories where a photoelectric colorimeter is not available.

[2] Dr. Julius Sendroy, Jr., has called to our attention the fact that alcohol, originally used in this method to aid in dissolving brom-phenols, also intensifies the yellow color. A more detailed discussion of principles for increasing the sensitivity of the photocolorimetric determination of iodate by means of the yellow color, based on independent observations of Sendroy and ourselves, will be made in another publication (Sendroy, J., Jr., and Alving, A. S., to be published).

the conditions of our procedure the sensitivities obtained with filters allowing maximum transmission at 400 and 365 mμ are approximately 20 and 65 times, respectively, greater than the sensitivity obtained with the No. 490 Evelyn filter (Fig. 1).[3] Plasma diodrast iodine for clearance studies can readily be measured with either of the more sensitive filters.

Our method makes practical accurate colorimetric determination of iodides or diodrast, by direct measurement of the yellow iodine color, in concentrations that previously could be estimated colorimetrically only by the blue color of the starch-iodine complex. Colorimetric analysis with the yellow color has the advantage of much greater speed and simplicity (4).

Reagents—

Bromine reagent. 10.0 gm. of reagent grade potassium bromate and 80.0 gm. of anhydrous reagent grade sodium bromide (iodide allowable as impurity, less than 0.01 per cent) are dissolved in 100 cc. of distilled water in a bottle. To this mixture 40.0 cc. of reagent grade 85 per cent phosphoric acid (H_3PO_4, sp. gr. about 1.7) are added from a volumetric pipette with occasional shaking of the bottle. This reagent should stand for at least a day before it is used to insure that the potassium bromate is completely reduced. When stored in a glass-stoppered bottle, the reagent keeps almost indefinitely.

Phenol reagent. To 100 cc. of 95 per cent ethyl alcohol in an Erlenmeyer flask are added 5.0 gm. of fresh reagent grade phenol, which must show no brown discoloration due to oxidation, and

[3] The No. 490 Evelyn filter concentrates 95 per cent of the light transmitted between 465 and 530 mμ, the approximate mean wave-length of the transmitted band being 490 mμ. The No. 400 Evelyn filter concentrates 95 per cent of the transmitted light between 380 and 430 mμ, the approximate mean wave-length of the transmitted band being 400 mμ. Intermediate sensitivities are obtained with Evelyn filters No. 420 and 440. The Evelyn photoelectric colorimeter and filters may be obtained from the Rubicon Company, Philadelphia. The special filter made of "red ultra" glass No. 584, 8 mm. thick, transmits 30.8 per cent of the light at 365 mμ (see "Addendum"). It is manufactured by the Corning Glass Works, Corning, New York. The range of transmission is from 334 to 404.7 mμ. When the Corning filter is employed, it is desirable to use 10 volts to supply current for the colorimeter lamp. Unless extra voltage is added to the 6 volt battery supplied with the Evelyn instrument, the "fine adjustment" of the galvanometer becomes so insensitive as to be practically useless.

10.0 cc. of 17 per cent phosphoric acid. The 17 per cent acid is prepared by diluting reagent grade 85 per cent phosphoric acid 1:5 with distilled water. The phenol reagent should not be kept more than 3 days.

Potassium iodide. 50 gm. of reagent grade potassium iodide are dissolved in 100 cc. of approximately 0.01 N sodium hydroxide. This reagent can be kept for many months, because the alkali prevents atmospheric oxidation of the potassium iodide.

Acid cadmium sulfate solution. 13.0 gm. of reagent grade cadmium sulfate ($3CdSO_4 \cdot 8H_2O$) and 63.5 cc. of exactly 1 N sulfuric acid are diluted to 1 liter with distilled water.

1.10 N sodium hydroxide. Reagent grade sodium hydroxide is used for this solution.

Procedure

Precipitation of Blood and Urine Proteins—The method of Fujita and Iwatake (5) is employed. To 1 volume of plasma or urine are added 8 volumes of the acid cadmium sulfate reagent, followed by 1 volume of 1.10 N sodium hydroxide. The mixture is agitated gently while the sodium hydroxide is being added, and is then shaken vigorously for a few seconds. After standing at least 30 minutes, the mixture is filtered and the filtrate diluted, if necessary, to contain between 1 and 5 γ of diodrast iodine per cc. Precipitation of protein must be made *before* the samples are diluted. The same filtrates can be employed for diodrast analysis as are used in the colorimetric determination of inulin (6),[4] because neither yeast fermentation nor the presence of inulin influences the final color development. If urine contains no protein, or only a trace, analysis may be made on the diluted urine without precipitation.

Oxidation of Diodrast Iodine to Iodate—The entire colorimetric analysis is carried out in the special "S" absorption test-tube (7 × 7/8 inches) furnished with the Evelyn photoelectric colorimeter (7). To 5 cc. of appropriately diluted filtrate is added 1 drop (0.03 to 0.05 cc.) of the bromine reagent.[5] The tube is gently shaken to obtain uniform distribution of the bromine, and is then

[4] Alving, A. S., Flox, J., Pitesky, I., and Miller, B. F., to be published.

[5] To insure reproducibility in the size of drops we reserve one pipette for the addition of bromine reagent and another for the addition of potassium iodide.

placed, unstoppered, in a bath containing vigorously boiling water. The water should extend slightly above the level of the liquid inside the tube.[6] After the tube has been heated for exactly 3 minutes, it is removed and immediately plunged into cool water and brought to room temperature. The outside of the tube is carefully dried before it is placed in a test-tube rack.

Removal of Excess Bromine—5 cc. of the phenol reagent are added from a volumetric pipette in the following manner: First, with the tip of the pipette held just above the surface of the solution in the tube, the reagent is allowed to run in freely until the solution has become clear and colorless. This requires less than 3 cc. The reagent remaining in the pipette is then used to wash down the inner walls of the tube. After the addition of the phenol reagent the tube must be carefully shaken to obtain a homogeneous solution. When mixing is completed, no schlieren figures are visible on the walls of the tube.

Development of Color—1 drop (0.03 to 0.05 cc.) of potassium iodide reagent is next added to the solution in the absorption tube.[5] The tube must be shaken immediately. Because the intensity of the resulting yellow color increases with time, in performing a series of analyses we add the reagent as follows: First, 1 drop of potassium iodide reagent is added to each tube containing a water blank[7] at intervals of exactly 30 seconds. 60 seconds are allowed to elapse after the addition of potassium iodide reagent to the last water blank.[8] The potassium iodide reagent is then added to the remaining tubes at intervals of 15 seconds. Other time intervals may be employed if found more convenient by the individual analyst.

It is best to measure the color intensity of all samples, including water blanks, *vide infra*, as close to 10 minutes after the addition of potassium iodide as possible. Longer periods may be employed

[6] It is convenient to analyze as many as thirty-four tubes at a time when the circular metal rack designed for the analysis of inulin is used. We obtained our rack from E. H. Sargent and Company, Chicago.

[7] By water blank is meant a sample containing reagents and 5 cc. of distilled water. The plasma blank consists of reagents and 5 cc. of a 1:10 protein-free filtrate of a sample of plasma obtained prior to the intravenous injection of diodrast.

[8] This interval allows adequate time for subsequent adjustment of the center point on the galvanometer scale.

but a more intense light source then becomes necessary and the value of the plasma blank,[7] as well as the urine blank, increases.

Estimation of Color Intensity—The light transmission is measured in an Evelyn photoelectric colorimeter (7) with a No. 400 Evelyn filter, or a special Corning filter having maximum transmission at 365 mμ.[3] Although considerably greater sensitivity may be obtained with the latter filter (Curve B, Fig. 1), the former

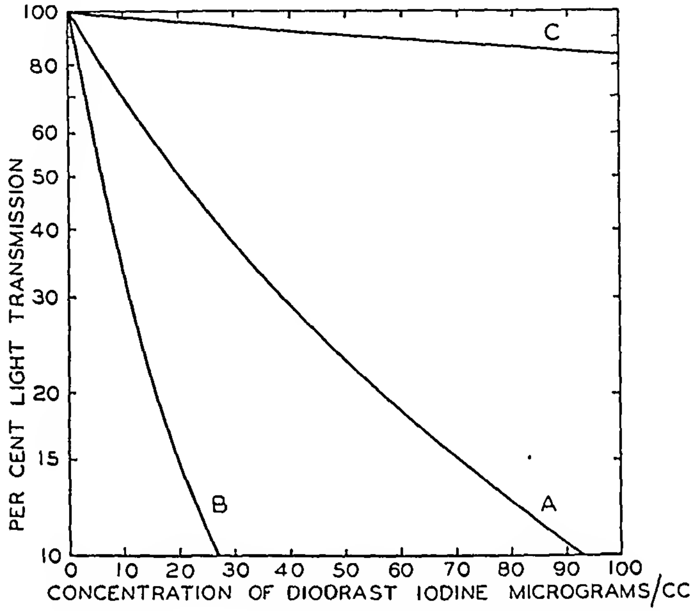

FIG. 1. Calibration curves showing the relation between the per cent of light transmitted (Evelyn photoelectric colorimeter) and the concentration of diodrast iodine with filters having maximum light transmission at 400 mμ (Curve A), 365 mμ (Curve B), and 490 mμ (Curve C).

is preferred for routine analyses in diodrast clearance studies (2) because it is sufficiently sensitive and allows greater analytical range (Curve A, Fig. 1). The diodrast iodine concentration is determined from a calibration curve.

Individual samples must be examined for color intensity in the Evelyn instrument in the same order and at the same time intervals between individual readings as were followed in the addition of the potassium iodide reagent.

Determination of Center Point Setting of Evelyn Galvanometer—

In each series of analyses water blanks must be included. To prepare blanks, 5 cc. of distilled water are substituted for the filtrate of plasma or urine. The water blank is treated in exactly the same manner as described above for filtrates. The photo-electric colorimeter is adjusted to read 100 per cent light transmission with a tube containing the water blank in the instrument. The water blank is then removed and the galvanometer reading, without any tube in place, is taken as the center point setting. It is advisable to run water blanks in triplicate to determine an average center point setting.

Calibration—Standards containing from 0.175 to 8.75 γ of diodrast iodine per cc. are prepared by dilution of 35 per cent diodrast.[9] 5 cc. samples of the standards are analyzed as described for filtrates. Water blanks must be included for center point setting. The per cent light transmission, obtained from the Evelyn colorimeter, is plotted against concentration of diodrast iodine on semilogarithm paper as shown by the calibration curves in Fig. 1.

Blank Analyses of Blood Plasma and Urine—The rate of color augmentation after the addition of potassium iodide is slower in water solution of diodrast than in filtrates of plasma containing diodrast. However, the water solutions and plasma filtrates increase in color at the same relative rate as do their respective blanks over a considerable period of time. It is, therefore, necessary to correct the plasma diodrast values by subtracting the value of a plasma blank,[7] which has been treated exactly as an unknown and analyzed simultaneously. The plasma blank is always determined on 1:10 filtrates, while the unknown samples may have to be diluted further. In such instances the blank must be subtracted only after both the plasma blank and the unknown samples have been multiplied by their respective dilution factors.

The difference in the rates of reaction, described above, causes the plasma blank to increase in magnitude with time. In order to keep the plasma blank low, it is desirable to read the color intensity of all samples, including water blanks and plasma blanks,

[9] Diodrast is the trade name for the brand of 3,5-diiodo-4-pyridone-N-acetic acid diethanolamine made by the Winthrop Chemical Company, Inc. It has, according to the manufacturers, a molecular weight of 510 and contains 49.8 per cent iodine.

as soon as practical after potassium iodide is added. As already stated in the discussion of the measurement of color intensity, we have found the ideal time interval before reading is made to be about 10 minutes. During a period of less than 10 minutes the color intensity changes too rapidly for the convenient measurement of light transmission; and, if a long interval is allowed to elapse, the plasma blank becomes large. The plasma blank is usually equivalent to about 0.6 mg. of diodrast iodine per 100 cc. if read in 10 minutes.

The intensification of yellow color that occurs after the addition of potassium iodide to water blanks or to water solutions of standards is probably due to atmospheric oxidation. In the case of plasma filtrates, the more rapid color intensification is due to the presence of unidentified substances which after bromination react like bromoamines (8), in that they liberate iodine from potassium iodide at a faster rate than is accounted for by atmospheric oxidation.

The value of the plasma blank for a given person is not affected by meals. The blank is of the same order of magnitude in uncontrolled diabetic and uremic patients as in normal individuals, and for an individual remains remarkably constant for a period of at least 6 hours.

Although the value of the urine blank containing no diodrast is greater than that of the plasma blank, omission of the urine blank usually introduces considerably less than 1 per cent error, because diodrast is greatly concentrated during excretion by the kidney.

EXPERIMENTAL

Determination of Diodrast Added to Dog and Human Plasma and Urine—Diodrast was added to normal plasma and analyzed. The results given in Table I show the high degree of accuracy obtained with the method, even at plasma diodrast iodine concentrations as low as 1.0 mg. per 100 cc. Comparable recoveries were obtained with analyses performed on normal urine containing added diodrast. Representative results for urine are shown in Table I.

Diodrast was added to urine obtained from patients suffering from "nephrosis," obstructive jaundice, uncontrolled diabetes mellitus, or uremia. In each case the accuracy of the diodrast method was the same as for samples obtained from normal individuals.

Variability of Plasma Blank—To determine the error that might be introduced by changes in the plasma blank during clearance determinations, blank analyses were performed on six blood plasmas drawn at intervals of 15 to 60 minutes in each of three subjects. The largest deviation of any of these eighteen plasma blanks from the mean for a given individual was equivalent to

TABLE I

Recoveries of Diodrast Added to Blood Plasma or Urine

	Diodrast iodine added	Diodrast iodine recovered	Error
	mg. per 100 cc.	*mg. per 100 cc.*	*per cent*
Blood plasma	1.00	1.01	+1.0
		0.98	−2.0
	1.99	1.97	−1.0
		1.95	−2.0
	2.99	3.03	+1.3
		2.92	−2.3
	4.98	5.10	+2.4
		5.01	+0.6
	13.94	13.71	−1.6
	15.85	15.90	+0.3
		16.15	+1.9
	34.86	34.20	−1.9
		35.00	+0.4
	58.10	57.30	−1.4
		58.30	+0.3
	69.72	69.51	−0.3
Urine	34.86	36.20	+3.8
	174.3	173.0	−0.7
	348.6	346.0	−0.7
	697.2	702.0	+0.7
	3486.0	3443.0	−1.2

0.05 mg. of diodrast iodine per 100 cc. The standard deviation from the mean was, respectively, 0.020, 0.028, and 0.012 mg. per 100 cc. for each of the subjects. It is apparent that the error caused by variations in plasma blank is small.

Specificity of Method—The method is applicable to the determination of neoiopax and iodides as well as to diodrast. Hippuran and skiodan cannot be oxidized to iodate by bromine as herein described. It is, therefore, possible to use this method for the

analysis of diodrast, neoiopax, or iodides in the presence of hippuran or skiodan.

Iodeikon can be completely recovered from water solutions and from urine by our method. It does not, however, appear in filtrates of plasma when cadmium is used as the precipitating agent. No other protein precipitants have been tried. Only a very small fraction of isoiodeikon can be recovered from water solution.[10]

Effect of Various Protein Precipitants—Several protein precipitants have been tested. Trichloroacetic acid was found to be unsuitable, as only a fraction of the diodrast could be recovered in the filtrate. Somogyi zinc precipitation yielded slightly low values at 1:10 dilutions. Tungstic acid precipitation is suitable for diodrast but not for inulin analysis. For this reason cadmium precipitation was adopted in preference to zinc or tungstic acid precipitation.

Effect of Blood Anticoagulants—Potassium oxalate, sodium citrate, and crystalline heparin do not interfere with the analysis.

SUMMARY

A method is described for the determination of diodrast and iodides by a simple, rapid, direct colorimetric procedure which possesses great sensitivity. In this method diodrast iodine in urine or filtrates of blood plasma is oxidized to iodate by bromine; excess bromine is removed by phenol; elementary iodine is liberated by potassium iodide; and the yellow color of the free iodine, after intensification, is measured in a photoelectric colorimeter. Sensitivity in color absorption is achieved by the use of filters allowing maximum light transmission at either 400 or 365 mμ.

By this procedure plasma diodrast iodine concentrations as low as 1 mg. per cent may be determined with a high degree of accuracy.

Addendum—Since this article went to press we have found that the new melt of Corning "red ultra" glass No. 584 has different light transmission

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[10] Skiodan used in this work was donated by the Winthrop Chemical Company, Inc.; neoiopax by the Schering Corporation; hippuran, iodeikon, and isoiodeikon by the Mallinckrodt Chemical Works.

characteristics than the melt from which the special filter used by us was made. Fortunately, however, a 4 to 5 mm. filter made of Corning "violet ultra" glass No. 586 (Melt 156) is very similar to glass No. 584 (old melt) and may be satisfactorily substituted.

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PHOTOELECTRIC MICRODETERMINATION OF IODATE AND IODINE

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A photoelectric technique for the colorimetric measurement of iodate and iodine has recently been described (Sendroy, 1939). As in the visual method (Sendroy, 1937), the color measured may be the yellow of the iodine element or the blue of the starch-iodine compound, the choice being largely dependent on the amount of sample available for analysis.[1]

Although the *relative color intensity* of the blue compound is about 100 times that of the yellow iodine when measurements are made with an Evelyn (1936) instrument,[2] there are certain dis-

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[1] With an Evelyn (1936) macro photoelectric colorimeter, Sendroy's (1939) method requires a minimum volume of 6 cc. of a solution of a minimum concentration of about 0.9 milliequivalent per liter (0.66 mg.) for macroanalysis of iodine by *yellow color measurement*, and a minimum of 6 cc. of an approximately 0.009 milliequivalent solution (6.6 γ) of iodine for microanalysis by *blue color* measurement. Amounts of iodine between the two limits mentioned, after appropriate dilution, are measured by blue color readings. For such measurements as could be made with the micro colorimeter attachment, about 1 cc. of the above solutions would be sufficient.

[2] The *relative color intensity* of a substance in solution in one color system, referred to that in another, is here defined as the reciprocal of the ratio of the concentration in the first system to that in the second, *when the photoelectric galvanometer deflection for both is exactly the same.* The difference in the color systems compared may lie (*a*) in the light filters used (change in light absorption by the same solution without actual change in color or its intensity), (*b*) in the chemical composition of the two solutions (resulting in different intensities of the same color, or the production of

advantages in the measurement of the blue color. The blue color is less stable than the yellow and subject to greater variation owing to the operation of other factors, such as the color development temperature, etc. (Sendroy, 1937, 1939). The determination is more time-consuming, and each series of measurements must be calibrated against a standard solution. Analysis by yellow color readings, on the other hand, although heretofore requiring larger samples, is simpler, more rapid, and slightly more accurate. The color development is affected by relatively few factors and is so reproducible that a permanent standard calibration curve may be used for all measurements.

On the basis of more recent data in the literature indicating a value of 470 to 480 mμ as the region of maximum absorption for iodine in the presence of KI in dilute solutions, a No. 490 filter[3] was prescribed for measurements of yellow iodine color with the Evelyn instrument (Sendroy (1939) p. 612). Shortly thereafter, we observed that yellow iodine color absorption was much greater when other filters were tried. At that time work on other problems made it desirable and advantageous to make yellow color readings for iodine determinations at low concentrations ordinarily requiring blue color readings. The present study of exactly defined conditions under which further increases in yellow color sensitivity could be obtained was therefore undertaken.[4]

The experiments to be described indicate how, by change in filters and solvents, such increased relative color intensity, or color "amplification," may be effected. There is also described a

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different colors), or (c) in a combination of both factors. Thus, in the region of 30 per cent light transmission, at the same reading for both solutions, the concentration of iodine (read with a No. 600 filter) present as the blue compound in one solution will be approximately only 0.01 that of a solution in which the yellow color is read (with a No. 490 filter) (Table I).

[3] With the exception of the Corning Ultra series, the color filters mentioned in this paper refer to those supplied by the Rubicon Company of Philadelphia for use with the Evelyn photoelectric colorimeter. The numbers of the Evelyn filters correspond to the wave-lengths, in mμ, at which they allow maximum light transmission.

[4] The present paper is based on principles originally applied to determinations of diodrast by Flox, Pitesky, and Alving (1942), and of calcium and chloride by Sendroy (1941, 1942). The general application of these principles to the determination of iodate and iodine in biological fluids is a result of the collaboration of both laboratories.

simple modification of Evelyn's photoelectric colorimeter, to facilitate an extension of its range of readings to the near ultraviolet region.

EXPERIMENTAL

Calibration Curves for Correlation of Galvanometer Readings with Iodate or Iodine Concentrations in Different Yellow Color Systems—According to a previously outlined procedure (Sendroy, 1939), varying volumes of dilute solutions of KIO_3 were treated with 0.085 M H_3PO_4 and 5 per cent KI (freshly prepared), and diluted with water or other solvent to a final volume suitable for the range of color measured by each filter. *Blanks, identical with the samples in all respects, but with iodate omitted*, were used for setting the Evelyn instrument at 100 for the base-line reading. 10 cc. portions of the color samples were read with the contents of the tubes at 25° (±5°). Data for complete calibration curves of each of the color systems discussed or mentioned in this paper were based on the averages of the readings of from two to six experiments for each system.

The calibration curves of Fig. 1, plotted semilogarithmically, indicate the range of *relative color intensities* obtained when yellow iodine colors were read under various conditions different from those originally described (Sendroy (1939) pp. 612–615) and represented here by Curves A and J. Essential data for the curves of the more important color systems, to which further reference will be made in this and other publications, are given in Table I.

Although yellow color curves such as these have been found very reproducible in our laboratories, and hence reliable for use *under the same conditions as those under which they were derived*, the galvanometer readings and corresponding iodine values are given in Table I as a guide and not as an actual working tool. We have sometimes observed slight discrepancies between readings with different individual filters of the same optical characteristics, in the same instrument, and also between readings with the same particular filter in different instruments. To avoid errors arising from possible variations in filters and photoelectric cell systems, *calibration curves should be determined with the instrument and filters with which they are to be used.* From time to time, the particular curve used should be checked for a redetermination of two or three points. In general, for iodate or iodine analyses in

solutions of otherwise varying composition, the analyst should either establish new calibration curves in accordance with the principles outlined in this and a previous paper (Sendroy, 1939) or else prove that previously determined or available curves apply

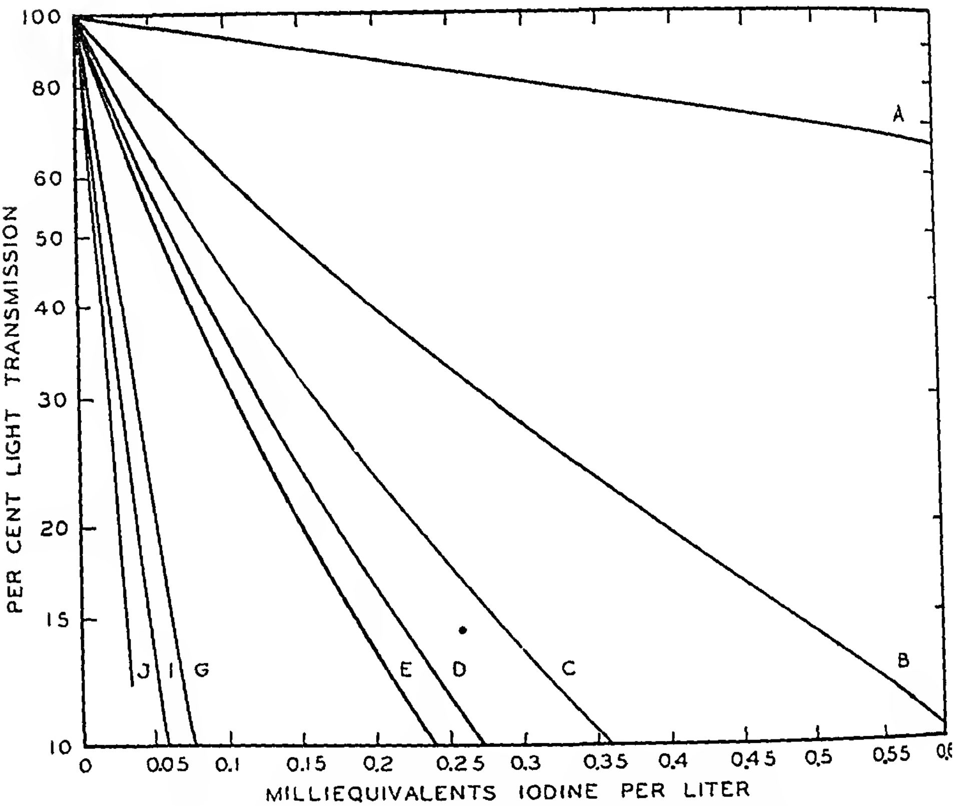

Fig. 1. Calibration curves relating galvanometer, or per cent light transmission, readings with iodine concentrations in samples analyzed in the Evelyn photoelectric colorimeter under various analytical conditions. Curves A, B, C, G, I, and J correspond to the color systems in Table I. Curves D and E correspond to systems like System C except that System D contained 1 per cent KI (instead of 0.06 per cent) and System E contained 40 per cent ethyl alcohol.

also to analyses of iodine in whatever particular solution it is desired to use them.

For practical use, it will be more convenient and accurate to plot the curves for each filter on a separate chart, with the abscissa scale so chosen as to avoid too steep a slope. Thus, for Curves

G, I, and J, the milliequivalents of iodine per liter should range from 0 to 0.1 in the same space used to plot values of 0 to 0.4 for Fig. 1.

TABLE I

Calibration Data of Curves for Photoelectric (Evelyn Colorimeter) Microdetermination of Iodate or Iodine, with Comparison of Relative Color Intensities, under Various Analytical Conditions

Color System...	A*	B	C	F	G	H	I	J†
Filter No.‡.....	490	420	400	584-8	586-5	586-5	586-5	600
KIO_3 solution, cc..........	1-7	0.5-13	1-10	0.5-10	1-9	0.5-7	0.5-7	0.5-10
mM..........	1.67	0.8	0.532	0.167	0.133	0.133	0.133	0.05
0.085 M H_3PO_4, cc..........	20	2.4	2.4	2.4	2.4	2.4	2.4	0.4
5% KI, cc.....	10	1.2	1.2	1.2	1.2	20	1.2	0.02
Other substances, cc. §							40 (C_2H_5OH)	1 (2% starch)
Galvanometer reading	Iodine concentration, m.eq. per liter							
60	0.73	0 099	0 058	0 0167	0.0160	0.0115	0 0111	0 0101
30	1.97	0 274	0.158	0 0437	0 0375	0.0282	0 0267	0 0197
15	3 43	0.480	0.277	0 0806	0 0606	0 0465	0 0433	0 0292
Relative color intensity, referred to System A, for readings between 20 and 40...........		7.4	12.4	46	54	70	74	106

* From a portion of Curve B, Fig. 1, of Sendroy (1939).
† From the blue color Curve B, Fig. 2, of Sendroy (1939).
‡ See foot-notes 3 and 5.
§ Water was added to 100 cc. volume in each case.

Effect of Variation in Light Transmission by Different Filters—When filters of the Evelyn series transmitting below 490 mμ in the *visible* portion of the spectrum were used, it was found

that, as the approximate mean wave-length of maximum light transmission of the filters decreased, galvanometer readings for the same solution also decreased (Curves A, B, and C, Fig. 1).

Since under these conditions no apparent peak of maximum absorption for yellow iodine solutions was found in the visible range, the Ultra group of the Corning Glass series (polished glass) was tested. These filters transmit light within a near ultraviolet range of maximum approximately 365 mμ, while absorbing the visible rays (No. 584, 5 and 8 mm., No. 585, 2.3 mm., No. 586, 5 mm. (Melt No. 156), No. 587, 5 mm., and No. 597, 5 mm.). Of this group, the filters No. 584, 5 mm., No. 584, 8 mm., and No. 586, 5 mm., in the order named, showed light absorption increasingly greater than that obtained with a No. 400 filter. Apparently, there is enough ultraviolet light transmitted from the lamp source to activate the photoelectric cell for measurements of low concentrations of iodine. Light transmitted by the other filters of this series was poorly absorbed by solutions of the same concentrations. Experimental details for readings with filters No. 584–8 and No. 586–5 are given in Table I, and the curve (Curve G) for the latter, in Fig. 1.[5]

The *relative color intensities* of iodine in Systems B, C, F, and G, referred to System A, given in Table I, indicate the increased sensitivity of measurement obtained when filters of transmissions of decreasing wave-lengths are used for the analysis of iodine in solutions of otherwise exactly the same composition. With the filter No. 586–5, the iodine concentrations of solutions analyzed by yellow color readings are only twice those of solutions analyzed by blue color readings.[6]

[5] The Corning Red Ultra No. 584 filter is made in stock polished thickness of 5 mm. The 8 mm. thickness used here was obtained from the manufacturer on special order. This filter transmits light within the near ultraviolet range of 334 to 404 (maximum 365) mμ. The Corning Violet Ultra No. 586 filter is made in stock thickness of 5 mm. It transmits light within the near ultraviolet range of 328 to 388 (maximum 360) mμ. Our filter was made from Melt No. 156.

[6] Of further interest is the fact that as the filter transmission maximum in mμ decreases the more nearly does the calibration curve approach a straight line. Even when plotted on a much larger scale, Curve G (Fig. 1) is a straight line, from the origin to a concentration of 0.06 milliequivalent of iodine per liter. Curve J, for blue color readings, is also a straight line, but does *not* pass through the origin (Sendroy (1939), Curve B, Fig. 2).

Effect of Solution Composition—By changing the composition of the solvent, there could be obtained a real or apparent increase in color of iodine determined with the same filter, as shown in the following experiments.

The effect of increased potassium iodide concentration is shown by a comparison of the relative color intensities of Systems C and D, and of Systems G and H (Fig. 1 and Table I, respectively). An increase of KI from 0.06 to 1.0 per cent, other conditions remaining the same for the two color systems compared, gave about 40 per cent more "effective" color in both cases. For System C, the addition of larger amounts of KI, to 40 per cent, resulted in still greater light absorption by the solutions, with an increase in *relative color intensity* to 26.0, or about 110 per cent more "effective" color.

The increase in "effective" color measured in these experiments was *not* a result of extra iodine produced by hydrolysis or photo-oxidation of the KI, for in each case the readings were made against an appropriate blank prepared simultaneously with the measured sample and containing the same concentration of KI. It was indeed observed that as time elapsed, the higher the KI concentration, the faster and greater was the actual production of free iodine, presumably by hydrolysis or photooxidation. Thus, for System C, when the blank for the 40 per cent KI tube was read against distilled water, the readings decreased from 75° to 35° in 20 minutes. However, throughout this period of rapid change of the blank, readings for a 0.16 milliequivalent iodine solution (against the same blank set each time at 100) were constant at 11, the production of extra iodine proceeding *pari passu* in both tubes, while the original increment in color remained constant. Because of these rapid changes, augmentation of "effective" color with increased KI beyond 1 per cent is not a practicable analytical procedure.

The effect of ethyl alcohol in the solvent is shown by a comparison of the color Systems C and E, and of Systems G and I (Fig. 1 and Table I, respectively). The effect of 40 per cent ethyl alcohol (40 cc. of 95 per cent alcohol, *filtered before use*, in 100 cc. of solution) was a little greater than that of 1 per cent KI, in the augmentation of "effective" color for light absorption at both 400 and 360 mμ.

In contrast to the effect of KI, there was little or no color developed in the blanks initially or on standing, the color values for all tubes being entirely stable for at least 1 hour.[7] Augmentation of "effective" color by addition of ethyl alcohol to 40 per cent by volume is analytically practicable.

Simple Modification of Evelyn's Apparatus for Readings in Near Ultraviolet—The use of near ultraviolet selective filters requires light of greater intensities than those originally provided for in Evelyn's (1936) apparatus. The necessary increased voltage imposes such a strain on the regular No. 31 (0.3 ampere, 6.2 volts, five cell) flashlight lamp as to limit its life to 1 or 2 days use. We have overcome this difficulty by the following changes in source of light and power supply for the Evelyn instrument made by Rubicon.

In place of the No. 31, there is mounted a No. 965 T $4\frac{1}{4}$ (0.5 ampere, 9.6 volts, eight cell) Mazda flashlight lamp.[8] The slightly larger size bulb and the possibility of increased heat effects necessitate the use of the adapter made of metal or opaque plastic, shown in Fig. 2. When the adapter is inserted in the receptacle usually holding the lamp reflector, the distance between the tip of the lamp bulb and the filter surface is the same as in Evelyn's arrangement. Also, the ventilation slots and the increased distance of filament from the filter tend to minimize increased heat generation effects. For power, supplied by a battery of storage cells, 10 volts should be used.

Since photoelectric fatigue and temperature effects are stated to be negligible in the original instrument (Evelyn, 1936), we have tested our modified arrangement for such possible effects. In actual use, this arrangement has shown extreme stability, with no sign of photoelectric fatigue. Heating may also affect readings by a change in temperature of the solution when read (Sendroy, 1939) or by changing the light transmission of the filter. Comparative tests with a No. 400 filter, under the original and under the modified conditions, have shown a difference of only 1° in

[7] When alcoholic solutions are poured into the color tubes, air bubbles may be formed, which, if allowed to remain during the readings, will lead to error. With gentle tapping, and on standing for a minute, such bubbles rise to the surface and disappear. The tubes containing the color solutions should be stoppered during the reading period.

[8] We purchased these lamps (Westinghouse) from Sears, Roebuck and Company.

the temperature rise of 10 cc. of water in a tube kept standing in the apparatus *for ½ hour*. For most readings, as usually made, this effect is negligible. Calibration curves obtained with various filters in the modified apparatus were sometimes slightly different from values previously determined with a 6.2 volt lamp. The difference in readings was one-quarter to one-half a scale division (0^1 or 0^2). This is probably a heat effect on light transmission through the filter. *For maximum accuracy, calibration curves and analyses should be obtained under the same conditions.*

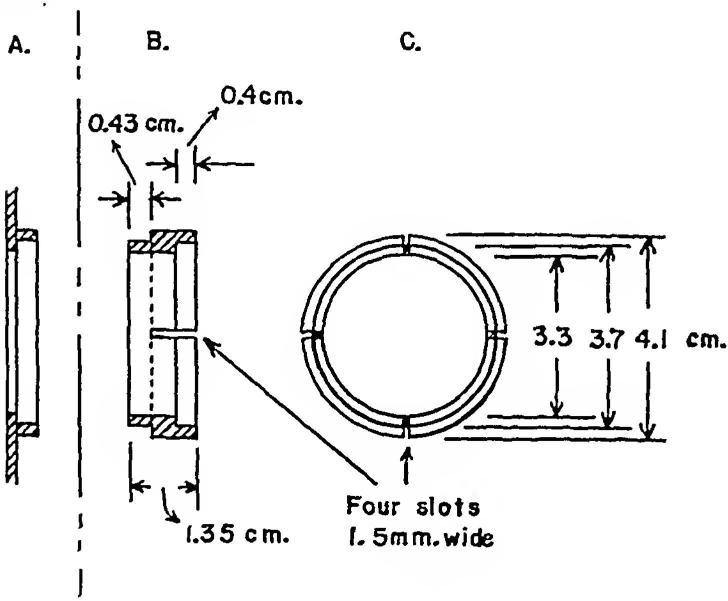

Fig. 2. Adapter for use of 9.6 volt lamp in the Evelyn (1936) photoelectric colorimeter; *A* cross-section of present receptacle for lamp reflector, *B* cross-section of adapter, and *C* front view of adapter.

The following additional observations have been made in practical use. The modified arrangement was suitable for readings with all filters from 720 to 360 mμ. The increased light intensity permitted the use of 6 cc. sample readings under conditions previously requiring 10 cc. samples.[9] The voltage drop

[9] Although we have not tested it with that purpose in mind, it seems quite possible that the modified arrangement described would be advantageous in the use of the *micro attachment* with highly selective filters calling for higher than macro voltages.

across the 9.6 volt lamp terminals was 6.2 volts with a 10 volt source (actually 9.2 volts), filter No. 586–5, "brighter" switch on; and 2.2 volts with filter No. 660, "brighter" switch off. The current drain for measurements through filter No. 400, with the 9.6 volt lamp and a 10 volt source, "brighter" switch on, was 390 milliamperes, compared with 260 milliamperes with the 6.2 volt lamp (no adapter used) and a 6 volt source, "brighter" switch on.

DISCUSSION

Of interest in connection with our work, the more important studies have been made by Martens (1902), Lachman (1903), Crymble, Stewart, and Wright (1910), Massol and Faucon (1917), Stewart and Wright (1917), Stobbe and Schmitt (1920), Julius, Ornstein, and Burger (1921), Brode (1926), Gróh (1927), Getman (1928), Batley (1928), Bovis (1928, 1929), Gróh and Papp (1930), Chirnoaga and Chirnoaga (1934), Walker (1935), Pamfilov and Teis (1936), Hock and Knauff (1936), and Cennamo (1939).

It appears that iodine colors, which vary throughout a wide range, from violet through red and brown to yellow, may be roughly classified into two groups, violet and brown. The *violet* colors, obtained when the solvent used has no dipole moment (C_6H_6, CCl_4, CS_2, hexane, etc.) have been found to yield spectra similar to that of the atomic vapor of iodine, with absorption maxima in the range of wave-length 500 to 520 mμ. The *brown* colors, on the other hand, obtained in "unsaturated" or "active" solvents having a dipole moment (H_2O, KI solutions, C_2H_5OH), have been found to yield spectra of "solvated" or molecular addition compounds of iodine and the solvent, with absorption maxima in the region of wave-lengths from 445 to 480 mμ. Peaks of maximum absorption have also been observed in the ultraviolet region, particularly at about 365 mμ.

The increased "effective" color with KI is probably the result of an increased production of the addition compound in the reaction

$$KI + I_2 \rightleftarrows KI_3$$

with resultant changes or shifts in the spectrum of iodine. The effect of ethyl alcohol, as in the case of KI, is probably a manifestation of "solvation" or molecular addition compound forma-

tion. For KI and alcohol solutions, the effects of which have been found very similar, most of the above authors have obtained a peak of maximum absorption at about 440 mμ (Martens (1902) gave a value of 420 mμ). Brode (1926) found no maximum absorption in the visible range for KI or alcoholic iodine solutions, but sharp peaks at 361 and 292 mμ.

Although, in agreement with Brode (1926), we have found no apparent maximum absorption peak for iodine with any of the filters used, but rather a continuous tendency toward increased absorption steadily from 490 to 360 mμ, there might be the possibility that such a peak, perhaps a small one, between 490 and 420 mμ, could be undetected in our photoelectric experiments. It is precisely within this region that most maxima have been reported. For this reason, we have begun an investigation, to be reported in the future, of the absorption curves of dilute iodine solutions under various conditions.

It will be sufficient, at this time, to state that preliminary results[10] for iodine in dilute KI solutions indicate no absorption peak whatever in the visible range from 600 to 400 mμ. Decreasing transmission is continued in the near ultraviolet region to approximately 355 mμ, where a definite minimum is found. The experimental results reported in this paper are in accord with such curves.

As for the primary purpose of this work, the object has been attained; namely, the extension of photoelectric yellow color readings for iodine to dilutions previously requiring analysis as the blue starch-iodine compound. In color System I the iodine concentrations at 30 per cent transmission are only about 1.4 times the concentrations read in the blue color System J (Fig. 1). Although each type of analysis presents its own problems and may not be susceptible to general treatment, it may be concluded that one technique, namely yellow color reading, will suffice for most analyses of iodine with the Evelyn photoelectric colorimeter.

SUMMARY

The photoelectric microdetermination of iodate and iodine (Sendroy, 1939) has been simplified and made more convenient by increasing the sensitivity of yellow color readings to a point ap-

[10] We are indebted to Dr. George I. Lavin and to Dr. M. N. States for these absorption curves.

proximating that of blue color readings in very dilute solutions. This has been done by the use of several selective filters efficient at the lower wave-lengths in the visible or in the near ultraviolet portion of the spectrum. A further increase in sensitivity was obtained by the addition of KI or ethyl alcohol to the solutions analyzed. A simple modification of Evelyn's (1936) photoelectric colorimeter, increasing the range of its readings to the near ultraviolet, is described.

We are indebted to Edward J. Fitzsimons for the major portion of the analytical work of this paper.

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NOTE ON THE PHOTOELECTRIC MICRODETERMINATION OF CHLORIDE IN BIOLOGICAL FLUIDS

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In the author's system of chloride analysis with silver iodate (Sendroy, 1937, 1939, *a*), colorimetric estimations of liberated iodine may be made by photoelectric measurements of yellow iodine color with a No. 490 filter, or of blue starch-iodine color with a No. 600 filter (Sendroy, 1939, *b*). In the preceding article (Sendroy and Alving, 1942) methods of increasing the sensitivity of yellow color measurements have been outlined.

For reasons discussed in the above papers, for analyses of chloride in protein-free filtrates of blood or serum, *blue color* readings have been prescribed only for the very rare occasions when 0.2 cc. or less of filtrate sample is available. However, even the smallest practical amount of supernatant fluid (0.1 cc., representing 0.01 cc. of serum, diluted to 20 cc. for color readings)[1] will be adequate for *yellow color* readings if a No. 420 filter is used. Analysis of the smallest samples by yellow color measurement with a filter of maximum transmission at lower wave-length than 490 mμ has the advantage not only of greater convenience but of eliminating the need for a filter (No. 600) which is not furnished as standard equipment with the Evelyn colorimeter. When there is a sufficiency of serum or blood filtrate, as will almost always be the case, and yellow color readings would be made as originally prescribed, an increase in sensitivity of such readings would seem to be superfluous. Nevertheless, here again, there is the practical

[1] Smaller samples of supernatant in the chloride analysis of serum or other fluids could be analyzed, when necessary, by the use of filter No. 400, or No. 586 Violet Ultra, 5 mm. thickness. Even greater increases of *relative color intensity* may be obtained by change in the solvents used for final dilution, as shown in the preceding paper.

TABLE I

Photoelectric Measurements with Filter 420 Compared with Titrimetric Results, in Analyses for Chloride by Silver Iodate Method

Material analyzed*		Chloride found, Titrimetrically	Chloride found, Photoelectrically†	
		mM per l.	*mM per l.*	*per cent deviation*
0.05 M NaCl	Dilution, 1:10 in 0.085 M H_3PO_4. For color, 0.5 cc. supernatant, 1.9 cc. 0.085 M H_3PO_4, 5 cc. H_2O, 1.2 cc. 5% KI, diluted to 100 cc.		50.2, 49.4 (49.8)	−0.4
0.075 " "			74.7, 75.0 (74.9)	−0.1
0.10 " "			99.5, 101.5 (100.5)	+0.5
0.125 " "			124.9, 124.8 (124.9)	−0.1
0.15 " "			152.5, 153.5 (153.0)	+2.0
Human‡ serum	Tungstic acid filtrate, 1:10. For titration, 0.5 cc. supernatant. For color, 0.5 cc. supernatant, 2.4 cc. 0.085 M H_3PO_4, 5 cc. H_2O, 1.2 cc. 5% KI, diluted to 100 cc.	106.1	105.2, 104.8 (105.0)	−1.0
		101.9	102.8, 101.8 (102.3)	+0.4
		103.0	103.4, 102.8 (103.1)	+0.1
		106.3	105.5, 104.8 (105.2)	−1.0
		106.7	106.7, 106.7 (106.7)	0.0
		105.1	105.5, 104.8 (105.2)	+0.1
Human§ blood	Tungstic acid filtrate, 1:25. For titration, 1.0 cc. supernatant. For color, 1.5 cc. supernatant, 0.9 cc. 0.085 M H_3PO_4, 5 cc. H_2O, 1.2 cc. 5% KI, diluted to 100 cc.	86.3	86.4, 85.0 (85.7)	−0.7
		91.7	91.7, 92.0 (91.9)	+0.2
		78.1	78.7, 79.4 (79.1)	+1.3
		80.9	78.7, 79.4 (79.1)	−2.2
		75.2	75.2, 75.2 (75.2)	0.0
		80.9	80.0, 81.4 (80.7)	−0.2
Average				±0.6

The figures in parentheses are averages.

* Dilutions of supernatant fluid for color readings were made in accordance with the outline for color System B, Table I, of Sendroy and Alving (1942). For samples of different sizes, addition of reagents and final dilution should be made exactly in proportion to the above.

† Values for color solutions, found photoelectrically from a calibration curve corresponding to Curve B, Fig. 1, of Sendroy and Alving (1942), *plotted in terms of mM of iodate per liter* (or from Curve B directly, and divided by 6), were used to calculate chloride (Sendroy (1939, *b*), Equation 3, p. 615).

‡ The blood samples were exposed to air, no precaution being taken to prevent migration of chloride from cells to serum.

§ See foot-note 2.

advantage of substituting the same filter (No. 420) which is provided as standard equipment for one (No. 490) which is not.

For the foregoing reasons, in the interests of convenience and simplicity, *all photoelectric measurements of chloride in serum and whole blood by the author's silver iodate method should be made with a No. 420 filter according to the color System B, outlined by Sendroy and Alving* ((1942) Table I).

Table I of the present paper indicates the details of technique, and the results obtained in the modified procedure for chloride analyses of salt solutions, serum, and whole blood,[2] when carried out in accordance with the above recommendation. Comparison with values for chloride known to be present or found by the titrimetric technique (Sendroy (1937, *b*) p. 411) indicates that the results are at least as good as those previously obtained with the No. 490 and No. 600 filters, as originally described.

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[2] It has occasionally been observed in this and other laboratories (Cullen and Leva, personal communication) that exceptional samples of whole blood, usually polycythemic, do not yield clear, colorless tungstic acid filtrates when deproteinized according to directions (Sendroy (1937, *a*) p. 354), although the ratio of the precipitant to blood is 15:1 as against 9:1 used by Van Slyke and Hawkins (1928). Apparently, the change in final dilution from 10 to 25 volumes, with consequent decrease in *strength* of the reagents, accounts for the inadequate deproteinization. The remedy in such cases is obviously an increase in the amounts of precipitant and acid used. As a matter of fact, the first two samples of whole blood in Table I were abnormal and required 18 cc. of tungstic acid reagent (instead of 15 cc.) and 0.68 M H_3PO_4 (instead of 0.34 M), for complete precipitation of proteins.

THE REACTIVITY OF PORPHYRINDIN IN THE PRESENCE OF DENATURED PROTEINS

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The estimation of sulfhydryl groups in native and in denatured proteins by titration with porphyrindin, a method first used by Kuhn and Desnuelle (10) and later developed and studied by one of us (6–8), has yielded much information concerning the distribution of these groups in the proteins. The advantages in the use of this dye for the estimation of mercaptan groups include (*a*) the rapid and stoichiometric reaction involved, (*b*) the lack of any effect on the dye by the presence of protein-denaturing agents (guanidine salts and urea derivatives), and (*c*) the fact that the reaction takes place at neutral pH. The disadvantages include (*a*) a long and costly synthesis of the dye, (*b*) a difficult visual end-point, (*c*) a loss in titer of the dye on standing in aqueous solution, and (*d*) the fact that the dye is reducible not only by mercaptan but also by tyrosine groups (4).

The first disadvantage has been partly overcome by simplifications in the synthesis introduced by Porter and Hellerman (12). The second disadvantage has been overcome by employing nitroprusside as an outside indicator; *i.e.*, by conducting the titration to the point where the —SH reaction with nitroprusside becomes negative. This method has been described in detail by Greenstein and Edsall (7) and has also been successfully employed by Anson (1) with various oxidizing agents. The third disadvantage has been obviated by standardizing the porphyrindin solutions against cysteine throughout the course of the titration with protein (6). In order to avoid the fourth disadvantage, the titration method was so devised (7) as to provide a stepwise and quite rapid estimation of the protein mercaptan groups, since the reaction of

porphyrindin with hydroxyphenyl groups is very much slower than with mercaptan groups. Inasmuch as the possibility nevertheless persisted that the tyrosine radicals interfered with the titration of protein mercaptan groups, it was considered desirable to investigate this problem further. For this purpose cysteine was added to solutions of denatured and in some cases previously oxidized proteins, and the cysteine so added titrated in the customary manner (7) with porphyrindin. The amount of cysteine recovered indicated that little or no interference by the non-mercaptan reducing groups in the proteins studied was encountered.

Procedure

Several aliquots of a neutralized solution of cysteine hydrochloride were added to solutions of various proteins in either 8 M guanidine hydrochloride or in 1 M glycine.[1] The cysteine solutions were standardized against iodine. The added cysteine was estimated in the presence of the denatured proteins by the stepwise procedure with porphyrindin, the titration being conducted to a negative nitroprusside end-point (7). The pH of the cysteine-protein mixtures varied from 6.4 to 6.8. The temperature was 25°. The time which elapsed between the addition of the cysteine to the protein and the beginning of the titration varied from 5 to 10 minutes. Titrations of cysteine in the protein solutions and in water alone with porphyrindin were conducted simultaneously. The reaction of the dye with cysteine alone was nearly instantaneous. The mixture of cysteine and protein, however, reacted more slowly with porphyrindin, and this rate of reaction was almost identical with that observed in the titration of mercaptan groups in denatured proteins. The time required for the added porphyrindin to oxidize completely the mercaptan groups present in a cysteine-protein mixture, or in a solution of denatured protein, was found to be approximately 30 seconds. Invariably, however, a full minute was allowed for this purpose. It is possible that the difference in the rate of titer of the cysteine in water alone and in

[1] Anson and Stanley (2) have rightly emphasized the necessity of using a pure grade of guanidine hydrochloride. The most satisfactory method of purifying this salt has been to dissolve it repeatedly in dry methanol at room temperature, and after filtering clear, to precipitate with an equal volume of anhydrous ether. The guanidine hydrochloride so obtained is neutral in reaction and free from possible oxidizing impurities (8).

the presence of the protein is due to the fact that, in the latter case, the mercaptan groups which are being titrated belong not to the added cysteine but to —SH radicals in the protein produced by reduction of the protein disulfide groups through action of the added cysteine.

The proteins used were of three types (6); namely, (*a*) amandin, in which sulfhydryl groups do not appear either in the native or in the denatured states of the protein (6), (*b*) horse serum albumin, in which sulfhydryl groups appear only in the denatured state of the protein (6), and (*c*) rabbit myosin, in which sulfhydryl groups are present when the protein is native, and either increase in amount when guanidine hydrochloride is added, or vanish when glycine and other ammonium salts are added (7).[2] Before the cysteine solution was added to solutions of serum albumin or of myosin in guanidine hydrochloride, the free —SH groups of the denatured proteins were exactly oxidized by the addition of the

TABLE I

Titration of Cysteine with Porphyrindin in Presence of Denatured Proteins

Protein	Denaturing agent	Dye added to oxidize protein —SH groups	—SH in denatured protein as cysteine	Cysteine added to solutions of oxidized proteins	Dye added to cysteine-protein mixtures	Recovery of added cysteine
		mM × 10³	*per cent*	*mM × 10³*	*mM × 10³*	*per cent*
Amandin, 1 cc. 5 0% solution in 5 0% NaCl	8 M guanidine HCl	0	0	8 0	3 9	97
				16 0	7 6	95
				32 0	15 8	98
Horse serum albumin, 1 cc. 4 9% solution	" "	0 16	0 08	8 0	4 0	100
				16 0	7 9	98
				32 0	15 2	95
Rabbit myosin, 1 cc. 1.0% solution in 0.5 M KCl	" "	0 19	1 19	8 0	3 7	92
				16 0	7 6	95
				32 0	15 4	96
" "	1 M glycine	0	0	8 0	4 0	100
				16 0	7 9	98
				32 0	16 0	100

[2] Horse serum albumin contains 4 65 per cent tyrosine (5). Myosin contains 3 38 per cent tyrosine (3). No data on the tyrosine content of amandin are available, but the protein gives an intense Millon reaction.

appropriate amount of porphyrindin. Cysteine was also added to a solution of native myosin in which the free —SH groups had been caused to disappear by the addition of glycine (7). Neither guanidine hydrochloride nor glycine interferes with the porphyrindin titration or with the selected end-point (6, 1).

The data are given in Table I.

DISCUSSION

The data in Table I reveal that the cysteine added to a solution of denatured protein can be nearly completely recovered by subsequent titration with porphyrindin. It is immaterial, apparently, whether the mercaptan titrated belongs to the added cysteine or whether it arises from disulfide groups in the protein which have been reduced to sulfhydryl by the added cysteine. In any event, there appears to be little or no interference by the non-mercaptan reducing groups of the denatured proteins when the method described (7) is closely followed.

Since the earlier investigations on estimating sulfhydryl groups in proteins by the use of porphyrindin were reported (6–8, 14), a number of similar studies employing a wide variety of oxidizing agents for sulfhydryl titrations have appeared in the literature (1, 2, 9, 11, 13). The data have been obtained on two well defined proteins, egg albumin and the tobacco mosaic virus, and are given in Table II.

With few exceptions, the data in Table II for each of the proteins studied show substantially good agreement. It must be remembered that one is dealing here as a rule with the oxidative titration by a variety of agents of autoxidizable groups in colloidal materials. Nevertheless, the results of Anson, Hellerman, and of Greenstein on egg albumin in guanidine and in duponol are nearly the same (Table II). Using the same denaturing agents, Mirsky obtained values for this protein somewhat lower than those obtained by the above investigators. The values of Rosner are the lowest of those given for egg albumin in Table II, and it may be that this is due to the fact that a substituting and not an oxidizing agent was employed for the titrations.

It is clear that the sulfhydryl group content of denatured proteins can be estimated by a wide variety of oxidizing agents. In the case of the tobacco mosaic virus all of the protein sulfur is accounted for as sulfhydryl sulfur (2). In the case of egg albumin,

about two-thirds of the cystine-cysteine sulfur is accounted for as sulfhydryl (6) (Table II). The sulfhydryl sulfur content of the denatured proteins studied so far has always been either less than

TABLE II

Sulfhydryl Groups of Denatured Proteins

Protein	Bibliographic reference	Denaturing agent	Titrating agent	—SH as cysteine
				per cent
Tobacco mosaic virus	Stanley and Lauffer (14)	Guanidine HCl	Porphyrindin	0.76
" "	Anson and Stanley (2)	" "	Ferricyanide, tetrathionate, or mercuric benzoate	0.71
" "	Stanley and Lauffer (14)	Urea	Porphyrindin	0.70
Egg albumin	Greenstein (6)	Guanidine HCl	"	1.28
" "	Anson (1)	" "	Ferricyanide, tetrathionate, or mercuric benzoate	1.24
" "	"	Duponol	" "	1.24
" "	Hellerman *et al.* (9)	Guanidine HCl	*o*-Iodosobenzoate	1.32
" "	Mirsky (11)	" "	Ferricyanide	0.96
" "	"	Duponol	"	0.96
" "	"	Urea	"	0.96
" "	Greenstein (6)	"	Porphyrindin	1.00
" "	Rosner (13)	"	Iodoacetate	0.87

or equal to the total cystine-cysteine sulfur of the proteins (2, 6–8) (Table II).[3]

[3] Egg albumin and the tobacco mosaic virus protein are two proteins whose —SH contents are revealed to nearly the same extent in urea and in guanidine hydrochloride. This does not mean that the two denaturing agents are necessarily equal in effect, since in the case of egg albumin the maximum —SH value is revealed at 8 M guanidine and at 16 M urea. In the case of myosin (7) 8 M guanidine hydrochloride liberates nearly twice as many —SH groups as does 16 M urea, and the effect of these agents on the physical properties of the protein is also in this order. The guanidine halides are much more powerful denaturing agents than urea (6).

SUMMARY

1. Cysteine was added in varying amounts to denatured proteins, and was nearly quantitatively recovered by subsequent titration of the mixtures with porphyrindin. This indicated little if any interference in the titration by other reducing groups of the proteins. The latter react too slowly under the conditions employed.

2. The data on the sulfhydryl group content of denatured tobacco mosaic virus protein and of egg albumin, obtained by the use of various oxidants and soluble denaturing agents, have been compared and discussed. In general, the results obtained by different investigators using diverse methods have been in substantially good agreement.

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THE LEAD CITRATE COMPLEX ION AND ITS RÔLE IN THE PHYSIOLOGY AND THERAPY OF LEAD POISONING

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Aub, Fairhall, Minot, and Reznikoff (1) in their classical study of lead poisoning introduced certain therapeutic principles which today constitute the accepted treatment of this disease. They suggested the use of calcium during the stage of acute toxicity in an attempt to remove the lead from circulation by depositing it in the bones, and the use of certain substances, notably acids and acid-forming salts, during the chronic phases, by means of which the deposited tertiary lead phosphate is converted to the much more soluble secondary phosphate, permitting its mobilization and excretion.

This latter phase of the treatment is not without its dangers, for mobilization of lead by the acid treatment has been known to cause the reappearance of toxic symptoms. In the light of present knowledge that in the case of calcium its physiological effects depend on the calcium ion rather than total calcium (2), it seems logical to assume that the lead ion is that fraction of the lead in the body which is responsible for the toxic manifestations of plumbism. Since a conversion from tertiary lead phosphate to the more soluble secondary phosphate entails an increase in lead ion concentration, it is seen why such a process occurring in the organism under the acid treatment might bring about the recurrence of toxic symptoms.

It has long been known in chemical analysis that sodium acetate will dissolve the relatively insoluble lead sulfate by the formation of a soluble, poorly dissociated lead acetate complex ion which lowers the concentration of lead ion to such an extent that its precipitation is prevented. It occurred to the author that this

phenomenon might be applicable to the problem of dissolving the insoluble lead compounds of the body tissues, making more lead available for excretion with no increase in lead ion concentration. A preliminary survey of the solubility of lead phosphate in dilute solutions of organic ions[1] disclosed the fact that, while the solvent effect of acetate, formate, ascorbate, and tartrate ions was not striking, citrate in very dilute solution (0.002 M) had a powerful solvent action on the insoluble lead phosphate.

The present work was undertaken in an attempt to determine the nature of the lead citrate complex and its degree of dissociation by measurement of lead ion activity in solutions containing lead and citrate ions.

EXPERIMENTAL

The lead ion activities were determined potentiometrically by the cell

$$Pb \mid Pb^{++} \mid \text{saturated } NH_4NO_3 \mid \text{normal calomel electrode}$$

That such a cell can be used satisfactorily to measure lead ion activity has been demonstrated by others (3–5). After a trial of numerous designs which included cells with amalgam electrodes, cellophane-covered electrodes, oxygen-free cells and solutions, and flowing liquid junctions, it was found that the cell described gave constant and reproducible potentials. The electrode consisted of a strip of pure lead coated with paraffin except for an area of about 2 sq. cm. at one end. These electrodes were cleaned immediately before use by immersion in nitric acid (1:4) for 3 minutes, followed by repeated rinsings in distilled water and final immersion in a solution identical with the one to be used in the cell. This treatment gave to the exposed area a uniformly crystalline, lustrous surface. The electrode was then immediately introduced into the solution contained in a glass concentration cell so that the exposed area was well below the surface of the liquid. This prevented the rapid oxidation of the lead at the interface between solution and air, and obviated the necessity for oxygen-free apparatus. The electrode deep in the solution did not oxidize appreciably until long after a constant potential had

[1] Kety, S. S., a paper read before the Undergraduate Medical Association of the Medical School of the University of Pennsylvania, April 4, 1940.

been reached. An inverted U-tube filled with the same solution as that in the cell and plugged with cotton at its distal end connected the cell to a saturated solution of ammonium nitrate into which the side arm of the calomel electrode dipped. Thus liquid junction was established without danger of contamination to the solution in contact with the electrode. The relative simplicity of this form of concentration cell made possible the simultaneous determination of solutions in batteries of four to eight cells.

The solutions of which the lead ion activity was to be determined were made by combining varying proportions of accurately standardized solutions of lead nitrate and the sodium salt of the organic acid. The pH of these solutions was determined potentiometrically by means of a glass electrode.

Equilibrium within the solution and between the solution and the lead electrode was usually attained within 30 minutes and the potential remained reasonably constant thereafter for several hours. Final readings were those which did not vary by more than 0.2 millivolt in the course of 30 minutes at a constant temperature of 25°. Measurements of electromotive force were made by means of a type K potentiometer.

As a preliminary to the investigation of the lead ion complexes, the lead ion activities in solutions of lead nitrate of different ionic strengths were determined in the cell described with the following formula,

(1) $$E = 0.4064 - 0.02955 \log a_{Pb^{++}}$$

where E represents the observed potential, and 0.4064 represents the difference between the standard electrode potential (E^0) of lead (0.1263 volt as determined by Carmody (6) and later designated by Lingane (7) as the most reliable in the literature) and the potential of the normal calomel half-cell at 25° (−0.2801). From these values for lead ion activity were calculated values for the activity coefficient of lead ion ($\gamma_{Pb^{++}}$) at various ionic strengths from the relation $\gamma_{Pb^{++}} = a_{Pb^{++}}/[Pb^{++}]$. These values for $\gamma_{Pb^{++}}$, since Equation 1 neglects the presence of an indeterminate liquid junction potential, have significance only in their relation to each other (assuming that the concentrated ammonium nitrate though it does not eliminate the junction potentials tends, nevertheless, to equalize them). The absolute values of $\gamma_{Pb^{++}}$, if indeed the

concept of individual ion activities has a basis in fact, remain indeterminate by present methods. Nevertheless, as will presently be shown, the relative values as calculated above and plotted in Fig. 1 serve a useful purpose in the determination of unknown lead ion concentrations.

In subsequent determinations each battery of cells contained at least one standard cell of known lead ion concentration (0.1 M lead nitrate) and calculation of the unknown lead ion concentrations was made with the use of a formula derived thus: From

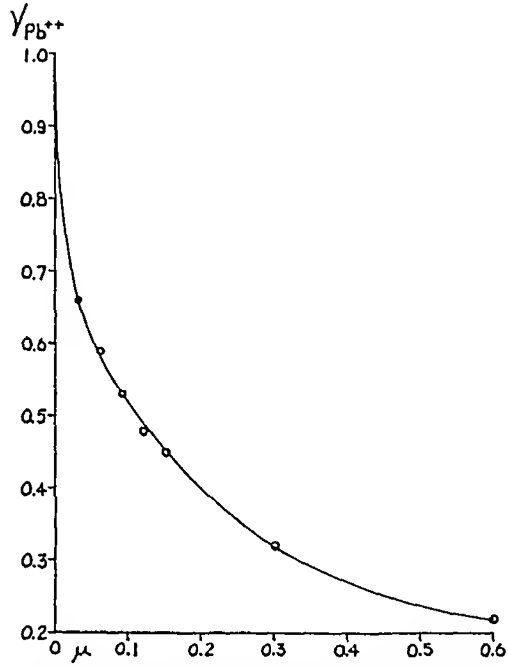

FIG. 1. Activity coefficients of lead ion in lead nitrate of varying ionic strength calculated from potentiometric data.

Equation 1, using for convenience the concept p denoting the negative logarithm of the term it precedes, introducing the liquid junction potential, E^j, and representing by x and s the unknown and standard solution respectively, we obtain

(2) $$E_s = 0.4064 + 0.02955\ \mathrm{p}a_{\mathrm{Pb}_s^{++}} + E_s^j$$

(3) $$E_x = 0.4064 + 0.02955\ \mathrm{p}a_{\mathrm{Pb}_x^{++}} + E_x^j$$

Assuming, as before, that the use of the ammonium nitrate salt bridge equalizes the liquid junction potentials, Equation 2 is

subtracted from Equation 3, resulting in the following rearrangement.

(4)
$$pa_{Pb^{++}_x} = pa_{Pb^{++}_s} + \frac{E_x - E_s}{0.02955}$$

But since $a_{Pb^{++}_{\mu a}} = [Pb^{++}] \times \gamma_{Pb^{++}_{\mu a}}$ at any ionic strength μa,

(5)
$$p[Pb^{++}]_x + p\gamma_{Pb^{++}_{\mu x}} = p[Pb^{++}]_s + p\gamma_{Pb^{++}_{\mu s}} + \frac{E_x - E_s}{0.02955}$$

whence

(6)
$$p[Pb^{++}]_x = p[Pb^{++}]_s + \frac{E_x - E_s}{0.02955} + p\,\frac{\gamma_{Pb^{++}_{\mu s}}}{\gamma_{Pb^{++}_x}}$$

where $p[Pb^{++}]_s$ in the case of the standard used equals 1, E_x and E_s are measured potentiometrically, and values for $\gamma_{Pb^{++}_{\mu s}}$ and $\gamma_{Pb^{++}_{\mu x}}$ are obtained from Fig. 1. Thus by the use of these standard cells factors such as the uncertainty of the E^0 for the lead and calomel electrodes, liquid junction potentials, impurities in the electrodes used, and variations in the potential of the calomel half-cell are minimized in the final result.

Results

In Fig. 2 are shown the changes in lead ion concentration resulting from the addition of increasing amounts of 0.1 M solutions of citrate, lactate, acetate, and ascorbate[2] ions to 0.1 M lead nitrate. In the case of citrate a precipitate (presumably Pb_3Cit_2) forms upon the addition of an equivalent amount of citrate which rapidly and completely dissolves in an excess of citrate. This alone is presumptive evidence for the formation of a poorly dissociated, soluble complex with lead. Citrate is unique among the ions studied in that it apparently forms a complex about 1000 times as effective as any of the others in its ability to bind lead.

The nature of the lead citrate complex is demonstrable from the following considerations. Assuming that its formula is the simplest possible, $PbCit^-$, and that in its dissociation

(7)
$$PbCit^- \rightleftharpoons Pb^{++} + Cit^{\equiv}$$

---

[2] The ascorbic acid used was provided by John C. Wyeth and Brother, Inc., Philadelphia.

it follows the mass action law, then

$$\frac{[Pb^{++}][Cit^{\equiv}]}{[PbCit^{-}]} = K_s \tag{8}$$

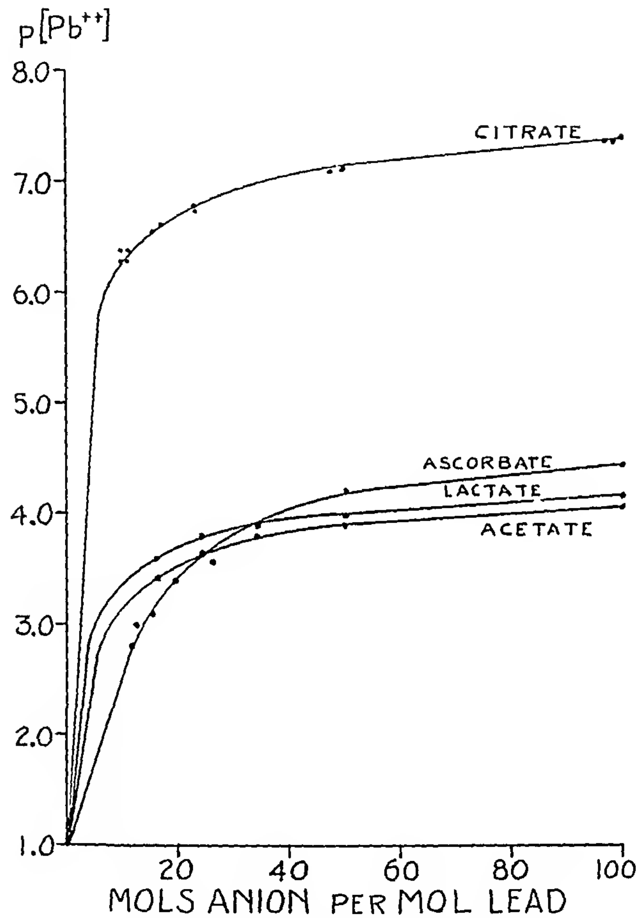

Fig. 2. Changes in lead ion concentration in 0.1 M lead nitrate upon the addition of sodium citrate, sodium ascorbate, sodium lactate, and sodium acetate.

where K_s represents the classical stoichiometric dissociation constant. Introducing the negative logarithm concept p

$$p[Pb^{++}] + p[Cit^{\equiv}] - p[PbCit^{-}] = pK_s \tag{9}$$

and rearranging, we obtain

$$p[Pb^{++}] = pK_s + p\,\frac{[PbCit^{-}]}{[Cit^{\equiv}]} \tag{10}$$

In Equation 10 it is possible to substitute experimentally deter-

mined values as follows: $[Pb^{++}]$ is calculated from potentiometric data by Equation 6; $[PbCit^-]$ is equal to [total lead] $-$ $[Pb^{++}]$, the latter being of such small dimensions that it may be disregarded, whence $[PbCit^-]$ = [total lead]; $[Cit^{\equiv}]$ is equal to [total citrate] $-$ $[PbCit^-]$, it having been established by means of the Henderson-Hasselbalch equation that at the pH of the solutions studied practically all of the citrate is in the form of the tertiary ion.

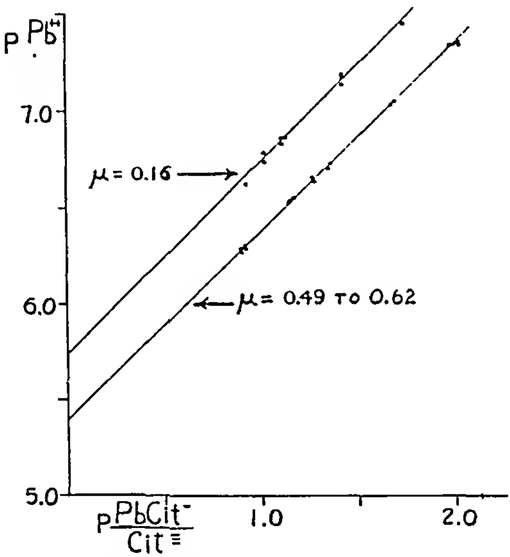

FIG. 3. Proof of the validity of the formula $PbCit^-$. Changes in lead ion concentration with changes in the ratio of $PbCit^-$ to citrate ion.

In Fig. 3 the validity of the assumed formula is tested. By plotting experimentally determined values of $p[Pb^{++}]$ against the negative logarithm of the ratio $[PbCit^-]/[Cit^{\equiv}]$ it is seen that at two levels of ionic strength straight lines are determined of slope 1, thus proving the validity of Equation 10 and establishing the formula of the complex as $PbCit^-$, with a dissociation which conforms strictly with the mass action law. It is of interest to note that this formula is in conformity with the complex ions $CaCit^-$, $MgCit^-$, and $SrCit^-$ established by an entirely different method

TABLE I

Values for pK_s of $PbCit^-$ Calculated from Potentiometric Data at 25°

Total lead	Total citrate	pH	μ	$E_x - E_s$	$-p\frac{\gamma Pb^{++}_{\mu s}}{\gamma Pb^{++}_{\mu x}}$	pK_s
mM per l.	*mM per l.*					
10.0	93.6	7.05	0.534	0.1605	0.140	5.37
10.0	93.6	7.05	0.534	0.1607	0.140	5.38
5.0	98.8	7.18	0.579	0.1716	0.155	5.38
5.0	98.8	7.18	0.579	0.1712	0.155	5.37
1.0	103.0	7.45	0.615	0.1921	0.168	5.33
1.0	103.0	7.45	0.615	0.1921	0.168	5.33
10.0	87.7	6.95	0.498	0.1594	0.124	5.38
6.0	92.6	7.12	0.540	0.1681	0.142	5.39
4.0	94.8	7.22	0.558	0.1737	0.148	5.37
2.0	97.0	7.35	0.576	0.1834	0.154	5.38
1.0	98.3	7.45	0.585	0.1923	0.158	5.36
10.0	86.8	6.95	0.492	0.1595	0.122	5.39
6.0	90.6	7.10	0.528	0.1678	0.138	5.39
4.0	92.6	7.22	0.546	0.1731	0.144	5.37
2.0	94.6	7.35	0.564	0.1829	0.150	5.37
1.0	95.5	7.44	0.573	0.1920	0.153	5.37

TABLE II

Values for pK_s of $PbCit^-$ Calculated from Potentiometric Data at 25°

At constant ionic strength 0.160, $p\frac{\gamma Pb^{++}_{\mu s}}{\gamma Pb^{++}_{\mu x}} = 0.134$.

Total lead	Total citrate	pH	$E_x - E_s$	pK_s
mM per l.	*mM per l.*			
0.5	27.0	7.43	0.1865	5.72
1.0	27.0	7.36	0.1776	5.73
1.0	27.0	7.35	0.1787	5.77
2.0	28.0	7.22	0.1687	5.73
2.0	28.0	7.22	0.1692	5.75
2.0	28.0	7.20	0.1692	5.75
2.5	28.0	7.16	0.1959	5.74
2.5	28.0	7.14	0.1672	5.78
3.0	28.0	7.10	0.1622	5.70
Average				5.74
Standard error				±0.008

(2) but the lead citrate complex ion dissociates much less readily. Values for pK_s of $PbCit^-$ precisely calculated from the experimental data are presented in Tables I and II. It is seen that in the pH range of 6.95 to 7.45 this pK_s is apparently independent of hydrogen ion concentration.

This dissociation constant does, however, change with ionic strength, which is to be expected of all stoichiometric mass action

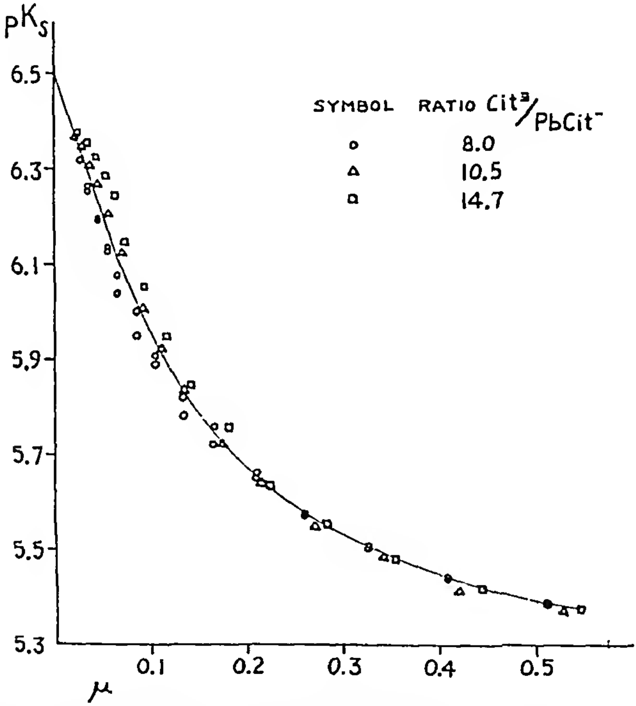

FIG. 4. Changes in pK_s of $PbCit^-$ with changes in ionic strength. Extrapolation to infinite dilution yields an approximate value of 6.50 for pK_a.

constants. The relation of K_s to the thermodynamic dissociation constant, K_a, may be expressed

(11)
$$K_a = K_s \frac{\gamma Pb^{++} \gamma Cit^{\equiv}}{\gamma PbCit^-}$$

whence

(12)
$$pK_s = pK_a - p\,\frac{\gamma Pb^{++} \gamma Cit^{\equiv}}{\gamma PbCit^-}$$

At any particular ionic strength the value of the ratio $\gamma_{Pb^{++}}\gamma_{Cit^{\equiv}}/\gamma_{PbCit^-}$ should be less than 1 and pK_s therefore less than pK_a. Further, at infinite dilution, where all activity coefficients are equal to unity, pK_s should equal pK_a. In Fig. 4 are shown the changes in pK_s with changing ionic strength. As the solutions became more dilute, measurements became increasingly difficult until at ionic strengths of about 0.03 the cells failed to maintain a constant potential. It is seen that the change in pK_s is in accord with prediction and by extrapolating the average curve to infinite dilution a value of 6.50 is obtained for pK_a. It should be pointed out, however, that the extrapolation is made from a part of the curve where experimental error is greatest; consequently the value for pK_a can be considered only a rough approximation.

DISCUSSION

Lead is known to exist in the body, like calcium, in non-diffusible and diffusible forms, of which only the latter is available for excretion through the membranes of the kidney and bowel. Without proof of the existence of soluble complexes of lead, the diffusible lead fraction must be thought to consist wholly of simple lead ion, the concentration of which is determined by the solubility products of the insoluble lead compounds present in the organism.

Proof of the existence and nature of the lead citrate complex just presented casts new light on the state of the lead in the body. By rearrangement of Equation 8 it is seen that

$$[Cit^{\equiv}] = \frac{K_{PbCit^-}[PbCit^-]}{[Pb^{++}]} \tag{13}$$

and similarly for calcium

$$[Cit^{\equiv}] = \frac{K_{CaCit^-}[CaCit^-]}{[Ca^{++}]} \tag{14}$$

Since in a solution in which both equilibria exist there can be but one value for $[Cit^{\equiv}]$,

$$\frac{[PbCit^-]}{[Pb^{++}]} = \frac{K_{CaCit^-}}{K_{PbCit^-}} \times \frac{[CaCit^-]}{[Ca^{++}]} \tag{15}$$

When the proper values are substituted for the dissociation constants of $CaCit^-$ and $PbCit^-$ at the ionic strength of blood serum,

as determined by Hastings and coworkers (2) and the author (Table II) respectively, we obtain

$$(16) \qquad \frac{[\mathrm{PbCit}^-]}{[\mathrm{Pb}^{++}]} = \frac{[\mathrm{CaCit}^-]}{[\mathrm{Ca}^{++}]} \times \frac{6.03 \times 10^{-4}}{1.82 \times 10^{-6}} = \frac{[\mathrm{CaCit}^-]}{[\mathrm{Ca}^{++}]} \times 331$$

The value for the ratio $[\mathrm{CaCit}^-]/[\mathrm{Ca}^{++}]$ in normal human blood serum is readily calculated as follows

$$(17) \qquad [\mathrm{Cit}^=] = [\text{total citrate}] - [\mathrm{CaCit}^-]$$

if the relatively insignificant amounts of citrate which are bound by magnesium and lead are neglected. By substituting this value for $[\mathrm{Cit}^=]$ in Equation 14 the following relation is derived.

$$(18) \qquad \frac{[\mathrm{CaCit}^-]}{[\mathrm{Ca}^{++}]} = \frac{[\text{total citrate}]}{K_{\mathrm{CaCit}^-} + [\mathrm{Ca}^{++}]}$$

The concentration of total citrate in normal human plasma has been found by others (8, 9) to average 0.12 mM per liter, while the calcium ion concentration in typical normal human serum may be taken from the values reported by McLean and Hastings (10) as approximately 1.3 mM per liter. By appropriate substitution in Equation 18 the ratio $[\mathrm{CaCit}^-]/[\mathrm{Ca}^{++}]$ is found to be 0.063, which in turn substituted in Equation 16 yields a value of approximately 20 for the ratio $[\mathrm{PbCit}^-]/[\mathrm{Pb}^{++}]$.

Hence the normal blood citrate is capable of keeping in solution and in presumably less toxic form an amount of lead 20 times as great as that which the solubility of the insoluble lead compounds of the body would otherwise permit. It thus appears, since only lead in soluble form should be available for excretion, that the citrate normally present in the blood constitutes an important physiological mechanism whereby the body rids itself of lead.

The relationships in Equation 13 imply also that any increase in citrate concentration in the blood or urine should demand a corresponding increase in PbCit^- with no increase in Pb^{++}, since the latter is determined by independent solubility constants. It has been adequately demonstrated by others (9, 11, 12) that the oral administration of citric acid and especially the alkaline citrates produces a great increase in the blood citrate level and in the urinary citrate excretion. It should follow then that the administration of citrates, by increasing the mobilization and excretion

of lead without increasing the concentration of toxic lead ion, would constitute an effective and safe therapy in plumbism.

SUMMARY

1. The citrate ion is shown to form with lead a soluble complex ion of the form $PbCit^-$. The lactate, acetate, and ascorbate ions also form complexes with lead which dissociate much more readily.

2. The stoichiometric dissociation constant (K_s) of the lead citrate complex ion is determined for ionic strength from 0.615 to 0.020 and at pH from 6.95 to 7.45 at 25°. At the ionic strength of blood (0.160) and in the physiological pH range the value of pK_s for $PbCit^-$ is 5.74 ± 0.008 standard error. An approximate value of 6.50 is obtained for the negative logarithm of the thermodynamic dissociation constant for $PbCit^-$.

3. Evidence is presented that the normal blood citrate constitutes a physiological mechanism for the removal of lead from the body.

4. It is suggested that the administration of citrates may constitute a safe and effective therapy in plumbism.

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ELECTROPHORESIS OF THE RABBIT PAPILLOMA VIRUS PROTEIN

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The results of preliminary studies (1) on electrophoresis of the rabbit papilloma virus protein (2) have been described briefly in a recent note and mentioned further in association with findings on sedimentation, diffusion, and viscosity measurements (3) on the protein. The electrophoretic homogeneity and average mobility of the papilloma protein have been investigated in the pH range accessible with respect to stability and solubility of the material. A full account of the experiments is given in the present paper. In addition a procedure is suggested for characterizing boundary sharpness or spread based on the standard deviation of the Svensson curve and yielding values having the dimensions of a mobility.

Material and Methods

For study of the electrophoretic behavior of the rabbit papilloma virus protein the moving boundary method of Tiselius (4) was employed. The apparatus made use of a standard 11 cc. rectangular glass cell. Concentration gradients at the boundary were photographed directly by means of a slightly modified Svensson (5) optical arrangement in which a straight filament tungsten lamp[1] of high intrinsic brilliance was substituted for the usual mercury arc, making possible the elimination of condensing lens and slit assembly. The advantages of the new system will be described elsewhere.

[1] A straight filament tungsten lamp having a suitable spring arrangement to keep the filament taut was supplied through the kindness of Dr. H. C. Rentschler of the Westinghouse Electric and Manufacturing Company, Bloomfield, New Jersey.

The virus protein was purified in the usual way by ultracentrifugation of extracts of cottontail rabbit warts (2) and dissolved in buffer solution. The buffer solutions were prepared by dissolving for each liter 2.051 gm. of sodium acetate, 5.152 gm. of sodium veronal, and 2.922 gm. of sodium chloride. In making the solution to volume, 5.0 N hydrochloric acid was included to give the desired pH. This system was chosen because it was desired to examine the papilloma protein over a wide range of pH in a single buffer system in which the protein was known to be stable and in which the ionic strength could be kept constant. Protein solutions were dialyzed at 0–5° with agitation and frequent changes of buffer until the conductivity of buffer and protein solutions became constant. Conductivity was measured at 0° in a specially constructed cell (6) by means of an audio frequency A.C. bridge and suitable oscillator. Protein concentration was then found by micro-Kjeldahl nitrogen analysis. The boundary was established between protein solution and buffer in the electrophoresis cell at 0°, and a constant potential of 70 to 100 volts applied at the reversible electrodes. Under such conditions a current of about 0.015 ampere was obtained. Current measurements to about 0.1 per cent accuracy were made with a potentiometer and repeated frequently during migration, as the E.M.F. of the batteries used was subject to a small regular decrease over a period of 24 hours. These measurements together with the cross-sectional area of the cell permitted calculation of E (volts per cm.) in the cell to be made with more than sufficient accuracy.

During the migration the concentration gradient curves were observed on the ground glass of the camera, their development and progress noted, and frequent photographs taken. In most cases several such curves were photographed on the same base-line in order to show more clearly the change in shape with progress. Mobilities were calculated as velocities of the bisecting ordinate of the concentration gradient curve which is the same as the 50 per cent concentration point on the concentration curve.

For low mobilities on the alkaline side of the isoelectric point migration was allowed to proceed 16 to 24 hours. On the acid side, where mobilities were greater, the boundary moved an equivalent distance in 6 to 10 hours.

Results

The pH range available for electrophoresis studies on the papilloma virus protein is determined by its stability and solubility (7). In judging the stability range, the criterion infectivity rather than molecular stability has been applied. As judged by sedimentation studies, relatively little change in the molecules occurs between pH 2.9 and 10.0 in time intervals significant for the present work. On the other hand, the pH region of the stability of infectivity is somewhat more restricted, lying in the

TABLE I

*Electrophoretic Mobilities and H Values of Papilloma Virus Protein in Buffer Solutions of 0.1 Ionic Strength**

pH	Protein concentration	Mobility ($U \times 10^5$ sq. cm. sec.$^{-1}$ volt^{-1})			Heterogeneity value ($H \times 10^5$ sq. cm. sec.$^{-1}$ volt^{-1})		
		Ascending	Descending	Mean	Ascending	Descending	Mean
	mg. per cc.						
3.77	4.5	3.85	3.61	3.73			
3.78	3.4	3.87	3.67	3.77	0.74	0.74	0.74
3.81	3.4	3.27	3.45	3.36	0.43	0.65	0.54
4.10	3.3	2.51	2.42	2.46		0.79	
5.00				0.00			
6.54	3.5	−0.91	−0.94	−0.93	1.46	1.44	1.45
6.56	2.7	−1.03	−1.04	−1.04	1.07	1.28	1.18
7.77	2.7		−1.49			1.65	

* See the text for the preparation of buffer solutions.

region between 3.5 and 8.0. In order to be reasonably certain that the results obtained represented the behavior of the intact virus protein, examinations were made only between the limits related to infectivity. The range was narrowed further by insolubility of the protein between pH 4.2 and 6.0. The pH values selected for study and the respective findings are shown in Table I. An approximate value corresponding to pH 5.0 is given also. This value was obtained in the previously reported (7) determination of the isoelectric point by means of the Northrop-Kunitz chamber (8) and collodion particles.

In Fig. 1 are photographs of the protein concentration gradient

curves of the boundaries seen at pH 3.78 and in Fig. 2 are comparable curves obtained at pH 6.56. The individual peaks in Figs. 1 and 2 represent separate photographs superimposed on the same respective base-lines. For calculation of mobilities corresponding to the various pH values, the curves were projected and traced at a magnification of approximately 4 times. The locus of points midway between the edges of these somewhat thick traced curves was then drawn to give the final gradient curve. A planimeter was used to measure the area, and the ordinate bi-

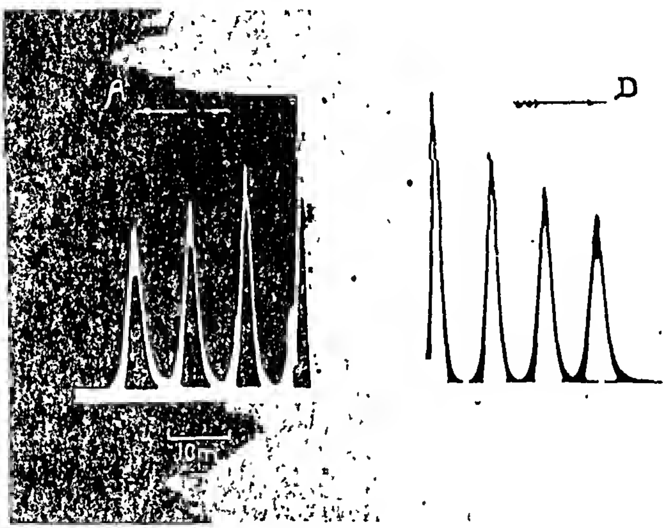

FIG. 1. Svensson diagrams of the rabbit papilloma virus protein migrating in 0.1 ionic strength buffer at pH 3.78. The time interval between successive peaks was 110 minutes. E = 3.26 volts per cm. D denotes the descending boundary migrating into protein and A the ascending boundary migrating into buffer.

secting the area was determined by trial and error with the planimeter. The abscissae corresponding to these ordinates were then used for mobility calculations.

The mobilities observed in the various pH regions are given in Table I and shown in Fig. 3 as functions of pH. At pH 4.1 and below the protein carried a positive charge, while at pH 6.54 and above the charge was negative. With the isoelectric point found previously with the Northrop-Kunitz cell, the smooth mobility curve shown was drawn by inspection.

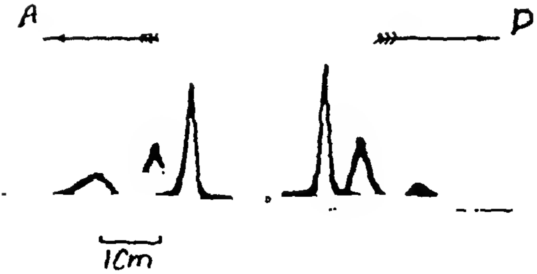

Fig. 2. *Svensson diagrams of the rabbit papilloma virus protein migrating in 0.1 ionic strength buffer at pH 6.56. The time interval between the first and second peaks was 421 minutes and that between the second and third was 682 minutes. $E = 2.4$ volts per cm. D denotes the descending boundary migrating into protein and A the ascending boundary migrating into buffer.*

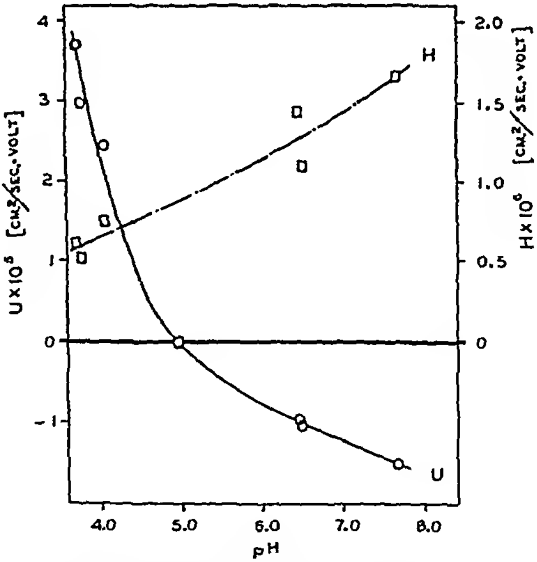

Fig. 3. Mobility and H values of the papilloma virus protein as functions of pH.

The information given by the findings with respect to homogeneity is of two sorts; namely, singleness of boundary and spread of boundary. In Figs. 1 and 2 the individual peaks are single. The same was found in the other studies, for on neither side of the isoelectric point, within the pH range studied, was boundary splitting observed. On the basis of this criterion, then, the protein exhibits high homogeneity on both sides of the isoelectric point. On the other hand, considerable differences were seen between the diagrams obtained on the acid side of the isoelectric point and those seen on the alkaline side. This was evident primarily in the degree of boundary sharpness, as exemplified in Figs. 1 and 2. At pH 3.77 to 4.1 the diagrams were very similar with respect to the concentration gradient at the boundary, as shown in Fig. 1. On the alkaline side of the isoelectric point, though the boundaries were single, there was apparent a considerable spread, as seen at pH 6.56 in Fig. 2. An analysis of the findings has been made to obtain an estimate of these curve differences in the two regions.

Since the protein examined here revealed a single boundary, the following considerations will concern only the spread of boundary during migration. In electrophoresis experiments, such as those made here, the resultant of various effects concerned with boundary spread is manifested in the form of the Svensson curve which portrays the distribution in the boundary of the individuals of the population of protein particles studied, regardless of the factors determining the distribution. In order to compare the curves of a single experiment or those of separate studies, it is necessary to have a means, preferably mathematical, for characterizing the distribution observed. The problem can be illustrated by comparison of the curves of Fig. 1 with those of Fig. 2. In the former the curves of both ascending and descending boundaries are sharp, and the progressive broadening of the curve with time seems slight. This sharpness is not evident in the latter, where the curves become flattened with progress of the two boundaries. For comparison of the curves of both boundaries in a single experiment and of the curves of different experiments, the problem becomes one of measuring boundary sharpness or spread and further, if possible, of correlating progressive boundary spread with factors known to be of influence.

Among the statistics furnishing information with respect to

the character of such curves is the standard deviation. For any single curve the standard deviation, σ, constitutes a value descriptive of sharpness or broadness and, under appropriate conditions, some idea of the similarity or difference between any two curves may then be made by comparing respective values of σ. In the present experiments it was observed that boundary spread was progressive with time. Further, assuming that boundary spread may be related to the electrophoretic behavior of the protein, it is possible that the progressive spread may be related to the electrical field applied. If boundary spread could be correlated with the measurable factors time and electric field, a means would be at hand for the comparison of individual curves and further of groups of curves on the basis not only of form but of the rate of change in boundary characters. A possible relation investigated in this respect is described in the equation $\Delta\sigma/E\Delta t = H$, in which $\Delta\sigma$ is the change in standard deviation between any two curves in a given sequence occurring during the time interval Δt in an electric field E. H represents a value determinable by experiment. The H value calculated in this way provides a criterion of possible usefulness for the description of boundary characters based on the rate of spread in unit electric field. The results of application of the relation are seen for the papilloma virus protein in Table I.

It should be noted that the H value of the descending boundary of a given experiment is closely similar to that obtained with the ascending boundary. This indicates a similar rate of spread of the two migrating boundaries in an individual experiment. This relation was evident in all experiments over the pH range investigated. Comparison of the findings in different experiments was of especial interest. In the experiment at pH 3.78 (Fig. 1), the average H value computed for the two sides from successive pairs of curves in the ascending and descending boundaries, respectively, was 0.74×10^{-6} sq.cm. per second per volt. At pH 6.56, where boundary spread seemed great from inspection, the average H value was 1.18×10^{-6} sq.cm. per second per volt, which is larger, though not greatly so, than that at the more acid pH. The increase in H appeared to be slowly progressive from pH 3.77 to 7.77. It should be noted that the curves of Fig. 1 were obtained over a period of $5\frac{1}{2}$ hours at pH 3.78, when mobility of the protein was high. In contrast, the curves of Fig. 2 were photographed

over a period of 18 hours and 23 minutes, since at pH 6.56 mobility was low and a much longer time was necessary for migration over a distance comparable with that of Fig. 1. An evaluation of the quantitative differences in spread under the two conditions would be difficult from inspection alone. However, despite the seemingly wide difference in the two sets of curves, comparison of the mean H values for the two experiments reveals an unexpectedly close similarity.

DISCUSSION

In the present studies, the papilloma virus protein has migrated with a single boundary within the limits of the pH region available for study of the intact material. In the acid region near pH 4.0, the boundaries have been not only single but of a sharpness comparable with that seen with hemocyanins (9) and crystalline proteins (10) of low molecular weight. On the alkaline side of the isoelectric point, though the boundaries were single, the spread, as judged by inspection, seemed much greater than that in the acid region. However, when the time factors involved in the studies in the two general pH regions were taken into consideration, it was found that the rate of boundary spread in the alkaline region was not greatly different from that in the acid range. This was seen in comparison of quantitative estimates of the rate of boundary spread per unit electric field in the pH regions studied as shown by $\Delta\sigma/E\Delta t$ or the H values given in Table I. From the acid to the alkaline regions of examination, H increased progressively, but the difference in the two regions was not great. It is of interest to compare the values obtained here with those of the purified pseudoglobulin GI (11), where H was considerably greater than any of the values observed here.

The results of these experiments may be regarded as evidence of a high degree of homogeneity of the papilloma virus protein purified by ultracentrifugation. This is seen in the singleness of boundary of the migrating protein and in the sharpness of boundary as revealed both by inspection and by the low values of H. These findings parallel the evidence of homogeneity furnished by the application of other criteria; namely, sedimentation, diffusion, and viscosity (3). Uniformity in the viral properties associated with the protein is indicated by the results of studies on infectivity, complement fixation, and neutralization (12).

Though a method of infectivity measurement of relatively high accuracy is available for the papilloma virus, findings concerned with this property, subject to recognized variation, can be construed only as indicative of relative uniformity in biological qualities and cannot be interpreted on the same basis as the results derived with the far more accurate physical and chemical methods. Within the limits of the criteria available, however, the present findings, correlated with those previously described, provide a basis for considering the papilloma virus protein to be a single molecular species.

With respect to the *H* value, it appears that, under proper conditions, the relation described may provide a useful criterion for the numerical estimation of boundary characters based on the rate of spread in unit electric field. As a measure of boundary sharpness, Tiselius and Horsfall (9) have suggested a method of analysis based on distance rather than time of boundary movement. This procedure has the disadvantage of yielding results which become discontinuous at zero mobility. The *H* value is independent of mobility and also of the sharpness of the starting boundary. It constitutes a simple measure of the total rate of boundary spread irrespective of the cause or summation of causes determining the spread, and has the dimensions of mobility.

For the above considerations *H* need bear no implications relative to the nature of the causes responsible for boundary spread. On the other hand, the character of the present findings and those obtained subsequently with another protein preparation, the pseudoglobulin GI (11) of horse serum, has suggested the possibility of a more fundamental significance of the *H* value. This will be discussed in detail in a separate report (13) where the present and additional data with the papilloma protein will be considered from this point of view.

This work has been aided by a grant from the Lederle Laboratories, Inc., Pearl River, New York, and by the Dorothy Beard Research Fund.

SUMMARY

Electrophoresis of the rabbit papilloma virus protein has been studied in the Tiselius apparatus. Protein concentration gradient curves were obtained which indicate rapid cathodic movement at

pH values lower than 4.1 and slower anodic movement above 6.54. Insolubility of the protein about the isoelectric point prevented moving boundary experiments in this region, but observations previously made by means of the Northrop-Kunitz cell have indicated the isoelectric point to be at about pH 5.0. This point appears to fit the smooth curve relating mobility to pH found in the present experiments.

A new method of analysis based on change in standard deviation of the Svensson curve and yielding values denoted as H has been proposed to characterize time-rate of boundary spread in unit electric field. The H values have the dimensions of mobility.

The papilloma protein migrates with a single boundary at all pH values in its range of solubility and biological stability. In the acid region near pH 4.0 the material is extremely homogeneous electrophoretically and is only slightly less so on the alkaline side of the isoelectric point.

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THE ELECTROPHORETIC PROPERTIES OF SERUM PROTEINS

I. NORMAL HORSE PSEUDOGLOBULIN GI

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The problem of the number and distribution of proteins in normal and pathological sera has received ever increasing attention. While several albumin and globulin fractions have been isolated from serum with methods based on fractional precipitation with salts, a more detailed analysis of the composition of untreated serum has been made possible only by the application of the newer physicochemical methods of ultracentrifugation and electrophoresis in the Tiselius apparatus.

Sedimentation diagrams of normal, untreated horse serum show evidence of two distinct components which have been correlated with serum albumin and globulin, respectively (1). Electrophoretic diagrams, however, exhibit several peaks which have been related to α-, β-, and γ-globulin (2–6) and, depending on the experimental conditions, to one or two albumin components. While the albumin moves with a sharp boundary on both sedimentation and electrophoresis, indicating a high degree of monodispersity, the globulins appear to be devoid of any such uniform behavior. The sedimenting boundary ascribed to globulin shows a much higher degree of spreading in whole serum as well as in preparations obtained by precipitation with half saturated ammonium sulfate (1), and there is seen an even greater spreading and considerable overlapping of the diffuse boundaries during electrophoretic migration of untreated serum. This non-uniform behavior is also evident in salting-out studies on whole serum as well as on the isolated fractions, the albumin precipitating within rather narrow limits of salt concentration, whereas precipitation of globulins

occurs within wide concentration regions of the salting-out agent (7–12). The problem of the isolation and identification of the globulins is further complicated by the occurrence of fractions insoluble at their respective isoelectric points in salt-free solution, the euglobulins (13, 14) which seem to be associated in largest amounts with the γ-globulin (12).

The question of the place these euglobulins occupy in fresh normal sera, and of their relation to the water-soluble pseudoglobulins, is at present still a matter of conjecture. While with certain immune sera specific antibody activity (15, 16) has been associated with euglobulin material,[1] it seems possible that euglobulins obtained from normal sera may represent modified pseudoglobulins produced as a result of *in vitro* purification. Thus they differ markedly from the pseudoglobulins in respect to molecular shape (17) and would be expected, therefore, to make their appearance in sedimentation analyses of whole serum if they were present in the amounts found upon purification. Their close similarity to irreversibly denatured pseudoglobulins (18) as well as their progressive accumulation upon prolonged dialysis of whole serum (19, 20) or upon reprecipitation of pseudoglobulin[2] may be regarded as indicative of the ease with which pseudoglobulins may be converted irreversibly into a protein of euglobulin characteristics.

In a previous paper from this laboratory the isolation and purification of three apparently monodisperse pseudoglobulin fractions of normal horse serum were described, according to the method of fractional precipitation with salt, under rigid control of external conditions (11, 12). Two of these, referred to as GI and GII, have molecular weights of about 170,000, which is in satisfactory agreement with the value of 164,000 determined by Tiselius (2) for electrophoretically isolated globulin fractions. In the present work electrophoresis studies have been made on one of these fractions, pseudoglobulin GI, with the object (1) of establishing the electrophoretic homogeneity of the protein in terms of the number of migrating boundaries and the degree of reversible

[1] According to recent work pneumococcal antibodies of horse serum may be associated with a component having the electrophoretic mobility of γ-globulin (4).

[2] Erickson, J. O., personal communication.

boundary spreading, and (2) of comparing the electrophoretic behavior of this material with that of the globulin components of whole serum on one hand, and of electrophoretically isolated globulins on the other. Such a comparison appeared to be of special interest in order to determine whether and to what extent the purification process yielding pseudoglobulin GI had introduced changes in the properties of the untreated protein.

In addition, the electrophoretic behavior of the native purified protein was compared with that of the protein obtained by apparently reversible denaturation from concentrated urea solutions (18).[3]

A similar study of other globulin and albumin fractions of normal horse serum will be presented later.

EXPERIMENTAL

Method and Measurements

Electrophoresis of the horse serum globulins in solution was observed by the moving boundary method of Tiselius (21). The apparatus, which will be described in a separate paper, was similar to that of Tiselius except for the optical arrangement which operated on the principle described by Svensson (22). Protein solutions in appropriate monovalent buffers were placed in contact with buffer in an 11 cc. rectangular migration cell. Refrigeration equipment maintained the temperature at 0° ± 0.1°. Constant electrical potential of batteries was applied by means of reversible silver-silver chloride electrodes. All experiments described in this paper were carried out at 0.1 ionic strength in order to minimize δ-boundaries and boundary anomalies such as those frequently seen at lower salt concentrations. In these experiments a potential of about 100 volts was applied, and the resulting ion current was 0.010 to 0.015 ampere, depending on pH and buffer composition.

Conductivity of buffer and buffer-protein solutions was measured at 0° by means of an audio frequency A.C. bridge with the conductivity cell immersed in the same thermostat as the migration

[3] The term "reversibly" denatured protein is used here not as an accurate description of the process but merely to denote that fraction which, following the reversal of the conditions used for denaturation, approximates most closely the properties of the native protein.

apparatus. Measurements on both conductivity and ion current during migration were accurate to about 0.1 per cent.

Boundaries were photographed frequently, giving curves on the photographic plate in which the abscissae were proportional to distance moved and the ordinates measures of refractive index gradient. Such curves are the same as those obtained by the Lamm scale method where scale line displacement is plotted as ordinate. The apparatus photographs these curves directly without any moving parts. Both boundaries, that ascending into buffer and that descending into protein, were photographed simultaneously on each plate.

In each experiment the boundary was moved into view by slowly injecting buffer solution into one electrode chamber by means of a syringe activated by clockwork. When both boundaries had emerged from behind the horizontal plates of the migration cell, the syringe was stopped and the voltage applied. At this point the gradient of refractive index was so great that only part of the curve for the exceedingly sharp starting boundary could be recorded, but boundary spread soon brought the crest of the curves into view. Frequent photographs were made, and base-line thickness was minimized by means of the third blade on the camera slit, as described by Svensson (22). Several such curves were recorded on the same set of axes in order to show boundary movement and progressive change in shape. For accurate determination of boundary shape it was necessary to make a separate photograph of each pair of curves and to eliminate the third slit blade which introduces small errors into the area measurements.

Analysis of data was made to determine the number of components migrating separately and the mobility of each. In cases in which only a single boundary appeared, the rate of boundary spread was calculated also. For mobility determinations it was necessary to enlarge by a factor of 4 the Svensson diagram, examples of which are shown in Figs. 1 to 4 and to trace them on plain paper. These tracings were then measured by means of a planimeter, and the bisecting ordinate was determined for each curve. Distances measured between these bisecting ordinates were used for mobility calculations. Such a procedure is necessary with slowly moving materials showing a high degree of boundary spread and some skewness, and even with use of this method the

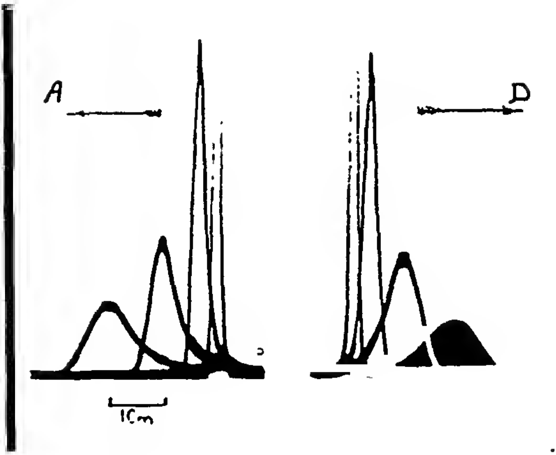

FIG. 1. Svensson diagram of pseudoglobulin GI migrating in 0.1 ionic strength buffer at pH 4.6. $E = 3.74$ volts per cm. D denotes the descending boundary migrating into protein and A the ascending boundary migrating into buffer.

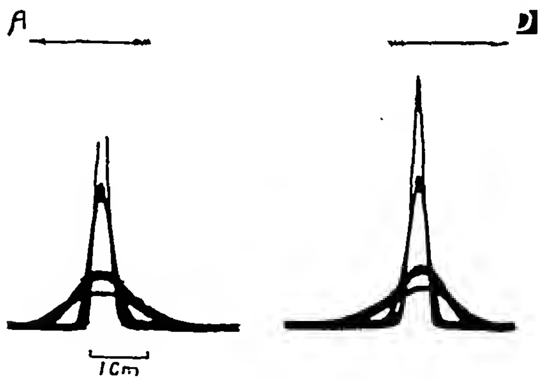

FIG. 2. Svensson diagram of pseudoglobulin GI migrating in 0.1 ionic strength buffer at pH 6.1. $E = 3.95$ volts per cm. D denotes the descending boundary migrating into protein and A the ascending boundary migrating into buffer. Although the maximum ordinate shifted slightly, no movement could be detected in the centroid of area. Note the considerable degree of boundary spreading.

small distances of migration make a high degree of accuracy difficult to obtain.

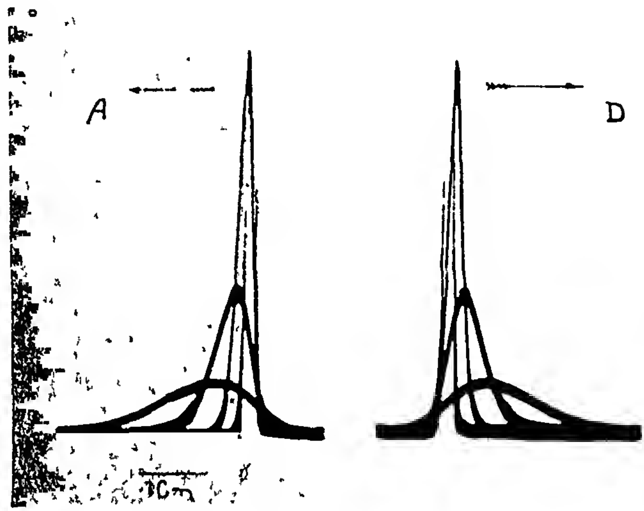

FIG. 3. Svensson diagram of pseudoglobulin GI migrating in 0.1 ionic strength buffer at pH 7.6. $E = 4.67$ volts per cm. D denotes the descending boundary migrating into protein and A the ascending boundary migrating into buffer.

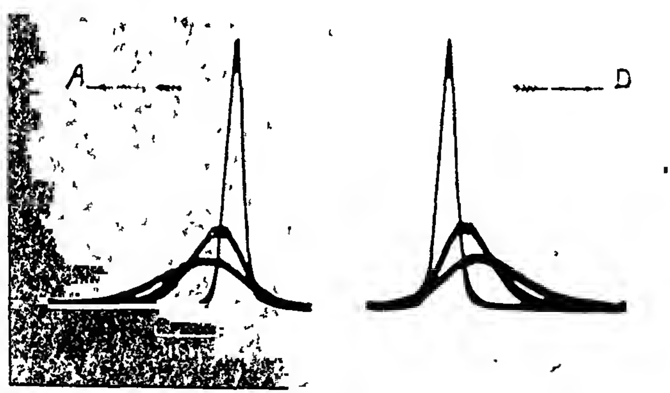

FIG. 4. Svensson diagram of pseudoglobulin GI "reversibly" denatured by 5 M urea migrating in 0.1 ionic strength buffer at pH 7.6. $E = 3.62$ volts per cm. D denotes the descending boundary migrating into protein and A the ascending boundary migrating into buffer.

Values of the mobility, U, were calculated in terms of conductivity of solution, C, ion current, i, and distance, d, moved by the centroidal ordinate per unit of time as follows:

$$U = \frac{CA}{i}\frac{d}{t} \text{ (sq. cm. per sec. per volt)} \tag{1}$$

In this equation A is the cross-sectional area of the migration cell and t the time of migration.

Measurements on rate of boundary spread of single boundaries were not affected by the small migration distances. This rate of spread can best be stated by means of a heterogeneity constant, H, described elsewhere (23, 24).

$$H = \frac{1}{E}\frac{\Delta\sigma}{\Delta t} \text{ (sq. cm. per sec. per volt)} \tag{2}$$

The fraction $\Delta\sigma/\Delta t$ is the rate of change of the standard deviation of the Svensson curve during migration. For its evaluation the individual curves of Fig. 1, for instance, were photographed separately without the third Svensson blade which thins the base-line. Enlargement was made as for mobility analyses and the bisecting ordinate determined. Second moments were then calculated about this axis[4] from measurements of the abscissae, x, at several evenly spaced ordinates, f. σ was then calculated directly by definition.

$$\sigma^2 = \Sigma f x^2/\Sigma f \tag{3}$$

The heterogeneity constant, H, was calculated for each of the several experiments, and the values are listed in Table I. The computations were made from successive pairs of curves in the respective ascending and descending boundaries in each experiment, and the H values tabulated represent the average for the corresponding group. Such analysis can be made with accuracy only in experiments in which ascending and descending boundaries are mirror images of each other. In cases in which this is not true, the lack of similarity is frequently due to gradients in pH and electric field at the boundary. An average value of H taken from corresponding ascending and descending boundary curves would then be a better measure of the electrophoretic heterogeneity of the protein ions.

[4] In theory the centroidal axis should be used for these measurements but the error introduced in these experiments by using the more conveniently determined bisecting ordinate is sufficiently small to justify the procedure.

Material

The protein was prepared from pooled normal horse sera in the manner described in detail previously (11). The pseudoglobulin GI was reprecipitated four times with ammonium sulfate from a 2 per cent protein solution at pH 6.4, at room temperature. The precipitates were collected from ammonium sulfate between 1.1 and 1.36 M and, before each subsequent precipitation, dialyzed and freed from the water-insoluble euglobulins by isoelectric precipitation at 0° (13). The yield of purified material was about 30 gm. from 4 liters of serum. The purified material was preserved by desiccation from the frozen state in an apparatus of the type described by Taylor and Beard (25).

TABLE I

*Electrophoretic Mobilities and Heterogeneity Constants of Horse Pseudoglobulin GI in Buffer Solutions of 0.1 Ionic Strength**

pH	Buffer	Mobility† ($U \times 10^5$ sq. cm. sec.$^{-1}$ volt^{-1})		Heterogeneity constant ($H \times 10^6$ sq. cm. sec.$^{-1}$ volt^{-1})	
		Ascending	Descending	Ascending	Descending
4.3	Sodium acetate-NaCl	+2.57	+2.29	3.38	5.12
4.6	Same	+2.23	+2.06	4.52	5.70
5.0	Sodium veronal-sodium acetate-NaCl		+0.76		
5.5	Same	+0.26	+0.29	8.00	7.26
6.1	"	0	0		6.69
6.8	Sodium veronal-NaCl	−0.77	−0.65	6.78	6.43
7.6	Same	−1.13	−0.98	7.70	7.05

* See the text for the preparation of buffer solutions.

† Computation of mobilities did not include consideration of δ- or ε-boundaries, since such boundaries were not present.

The denaturation of the protein by concentrated urea solutions and the isolation of reversibly denatured material will be described in full elsewhere (18). The globulins were denatured by 5 M urea solutions, and, after removal of the denaturing agent by dialysis, the irreversibly denatured fraction was precipitated at pH 6.0 and 0°. The supernatant solution was subjected to fractional precipitation with ammonium sulfate and the "reversibly" dena-

tured material collected between 1.1 and 1.36 M. After removal of traces of euglobulins by isoelectric precipitation, the protein was frozen and dried.

The buffer solutions were prepared as follows: For each liter of solution for use between pH 4.3 and 5.0, 4.101 gm. of anhydrous sodium acetate and 2.922 gm. of NaCl were weighed out. The salts were dissolved in a volume of distilled water slightly less than 1000 cc. To the solution was added the volume of 5.0 N HCl necessary to produce the desired pH, and the total volume was then increased to 1 liter. For pH 5.0 to 6.8, 2.051 gm. of sodium acetate, 5.153 gm. of sodium veronal, and 2.923 gm. of NaCl were similarly dissolved, an appropriate volume of 5.0 N HCl was added, and the total volume made to 1 liter. Between pH 6.8 and 7.6 a solution of 8.243 gm. of sodium veronal, 3.507 gm. of NaCl, and the requisite amount of 5.0 N HCl per liter volume was utilized. The ionic strengths of these solutions were thus essentially 0.1.

Protein solutions of 0.9 per cent concentration were made up in the desired buffer and equilibrated through cellophane membranes in the cold against three successive buffer portions under continuous stirring of both protein and buffer solutions. In each equilibration the buffer volume was 30 times that of the protein.

Results

In Fig. 5 the mobilities calculated from the descending boundaries and the average heterogeneity constants calculated from both ascending and descending boundaries are plotted as ordinates against pH values as abscissae. The isoelectric point of the protein studied here is near pH 6.0; the slope of the mobility curve in this region is $\delta U/\delta(\text{pH}) = 0.82 \times 10^{-5}$.

The heterogeneity constant is independent of pH in the region 7.6 to 5.5, and decreases somewhat below this pH value.

In Table I, the numerical values for mobility and heterogeneity constants are listed for both ascending and descending boundaries together with pH and buffer composition. Representative electrophoresis curves, as originally obtained by direct photography, are shown in Figs. 1 to 3 for the native protein and in Fig. 4 for the "reversibly" denatured protein. Comparison between the electrophoretic properties of the two materials is made in Table II. It may be seen that on the acid side the mobility of the "reversibly"

denatured protein is higher, and on the alkaline side lower than that of the native protein. It may be seen also that the values of H on ascending and descending curves are about alike from pH 5.5

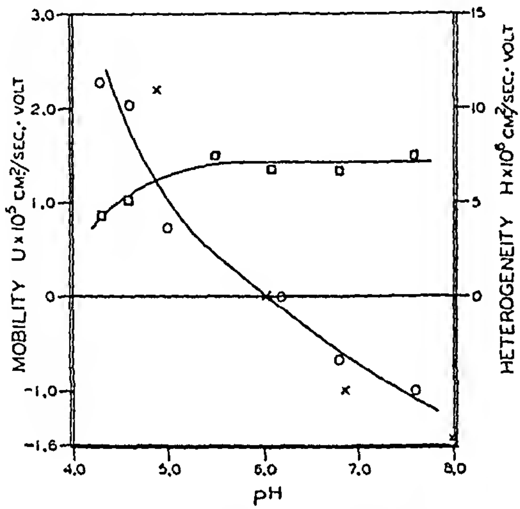

FIG. 5. Mobility and heterogeneity of pseudoglobulin GI in relation to pH at 0.1 ionic strength. The circles refer to the mobilities determined from the descending boundary. The squares refer to the average heterogeneity constant, H, determined from ascending and descending boundaries. The crosses are the mobility data of Tiselius (2) for the electrophoretically isolated γ-globulins.

TABLE II

Comparison of Electrophoretic Mobilities and Heterogeneity Constants of Native and "Reversibly" Denatured Pseudoglobulin GI

The values for H and U were calculated from the data of the descending boundaries.

pH	Native		Reversibly denatured	
	$U \times 10^5$	$H \times 10^6$	$U \times 10^5$	$H \times 10^6$
7.6	−0.98	7.4	−0.78	6.1
5.0	0.76		1.45	5.3

to 7.6. For pH values of 4.6 and less the ascending boundary has a lower H value and is sharper than the descending boundary. Nevertheless, both values of H and consequently the average are lower than those seen at higher pH values (Table I).

DISCUSSION

It may be seen from the data that the protein migrates with a single boundary over the entire pH range investigated. This may be regarded as a further criterion for the purity of this material which, as judged from diffusion and viscosity studies, appears to be monodisperse also with regard to the size and shape of the molecules (11).

The shape of the pH-mobility curve resembles that observed for many proteins, the mobility increasing rapidly as the positive charge on the protein molecule increases. The absolute values of the mobility are low as compared with the albumin fractions of normal horse serum (2).

Comparison of the present data with those obtained by Tiselius for the globulin fractions isolated with the method of electrophoretic separation (2) indicates the material studied here to be most closely related to his γ-globulin fraction. The observed isoelectric points are practically identical, while the mobilities of pseudoglobulin GI are somewhat lower on either side of the isoelectric point than those observed for the γ-globulin.

Although the migrating boundaries seen in Figs. 1 to 4 showed no evidence of splitting in the pH range examined, they were subject to a great amount of spreading which increased at a constant rate as the boundary moved. This effect was present not only at pH values at which migration was comparatively rapid but also near the isoelectric point (pH 6.1) where the average mobility was zero (Fig. 2). As a quantitative expression for the rate of boundary spread, the constant (24) H has been employed. It has the dimensions of mobility but, unlike U, it does not pass through zero. It does not become discontinuous at the isoelectric point. Values of H have been calculated from a series of curves such as shown in Figs. 1 to 4. The dependence on pH can be seen in Table I and in Fig. 5, where H is plotted against pH.

It is clear that, in general, diffusion and convection contribute in some measure to the boundary spread in moving boundary experiments. Nevertheless, for the pseudoglobulin GI in the pH region between 5.5 and 7.6, H is a measure of electrical inhomogeneity, for boundaries such as those of Figs. 1 to 4 are not only essentially mirror images of each other but, as will be described (24), regain most of their sharpness when returned to the starting

points by reversal of potential. This, together with the known monodispersity of the protein, indicates that H, in the region described, is a measure of the electrical properties of the pseudoglobulin GI. In the present experiment the values of H were approximately the same over a wide pH range, but in the pH region of rapid increase in mobility H became smaller. At all the pH values examined, however, H was of approximately the same order of magnitude as the mobility, U, indicating boundary spread through a distance comparable with that moved by the average particle.

Comparison of the data obtained on one hand with the native protein, and on the other with the protein obtained by "reversible" denaturation by 5 M urea, indicates a very close similarity between the electrophoretic behavior of these two materials when measured under otherwise identical conditions (Figs. 3 and 4). The degree of boundary spread of the "reversibly" denatured globubin is not greater than that of the native material, whereas the mobilities are different (Table II). Diffusion and viscosity measurements on these two fractions to be reported in detail elsewhere (18) indicate only minor differences in regard to size and shape. Thus the extended structure of the denatured globulin reverts rather uniformly to the condensed configuration when denaturation is "reversed."

The complex behavior of the serum globulins in regard to solubility and salting-out properties has been recognized for some time. This complexity is further emphasized by the high degree of reversible boundary spreading studied quantitatively in the present work and observed also with the material studied by Tiselius and others.

While chemical and physical measurements have furnished evidence for the presence of at least three major globulin components in normal sera, one observes a continuous gradation with respect to electrophoretic mobility. The distribution of mobility among the molecules shows three maxima which have been labeled α-, β-, and γ-globulin, respectively; however, there is considerable overlapping of the individual distribution curves into which the compound curve may be resolved.

The question arises, therefore, whether a similar continuous distribution may exist for such chemical and physical criteria as

carbohydrate and lipid content (26), molecular size, and molecular shape (11). Such a distribution would lead to the conclusion that the globulins represent a population of molecular species of closely interrelated properties. Salt precipitation or electrophoretic separation results in the isolation of a group of those molecular entities most closely related to each other with respect to the conditions chosen for their separation. Thus, it might be possible, for instance, to separate the serum globulins into an infinite number of fractions provided the increments in salt concentrations are sufficiently small. The physical and chemical properties of the individual fractions should then exhibit a similar gradation as has been observed for the electrophoretic mobility of the globulins as a whole.

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SUMMARY

The electrophoretic behavior of a pseudoglobulin fraction of normal horse serum, precipitable by 1.1 to 1.36 M ammonium sulfate at pH 6.4, has been investigated in the Tiselius apparatus with the Svensson optical system.

The protein designated as pseudoglobulin GI has been found to migrate with a single boundary over the pH range studied, the pH mobility curve being closely related to that observed by Tiselius for the γ-globulin fraction isolated by the method of electrophoretic separation. The degree of reversible boundary spreading, expressed quantitatively in terms of the heterogeneity constant, H, is great as compared with that of serum albumin. The H values are independent of pH between pH 7.6 and 5.5, and decrease below this region.

The electrophoretic pattern of the globulin "reversibly" denatured from 5 M urea is similar to that of the native protein, but the mobilities are higher on the acid side and lower on the alkaline side of the isoelectric point.

The high degree of reversible boundary spreading seen with the protein studied here as well as with the globulin components of whole horse serum is interpreted in terms of a continuous gradation in the electrophoretic properties of the globulin molecules. It is conceivable that a similar distribution may be obtained in regard to other physical and to chemical properties of the serum globulins.

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PROGRESSIVE BOUNDARY SPREAD IN ELECTROPHORESIS OF PROTEINS IN SOLUTION

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When solutions of mixed proteins are analyzed by the moving boundary method (1) in the usual type of Tiselius apparatus (2), it is found after a time that the sharp single boundary initially established has separated into several boundaries moving at rates characteristic of the several individual components of the mixture. If pure monodisperse proteins are examined, a single boundary is obtained no matter how far the motion continues. It is with solutions of such monodisperse proteins that this report is concerned.

In the analysis of purified protein, one observes not only continuous migration of a single boundary, but also a progressive broadening or spreading of the boundary with time. Much but not all of this broadening is lost when the electric field is reversed and the boundary returns to its original position. A number of factors have been supposed to influence the occurrence and degree of boundary spread. In the present paper these factors will be discussed in the light of experimental findings in studies on the rabbit papilloma virus protein and the pseudoglobulin GI of horse serum.

The optical system employed for the present experiments has been described in principle by Svensson (3). It produces at the photographic plate curves such as those of Figs. 1 to 6 whose abscissae measure distances in the cell parallel to the direction of boundary migration and whose ordinates are proportional to the gradients of refractive index in the boundary.[1] If a linear rela-

[1] The authors are indebted to Dr. Florence Seibert of the Henry Phipps Institute, University of Pennsylvania, Philadelphia, for much helpful information in the design of the Svensson slit used in this apparatus.

tionship is assumed to exist between refractive index and protein concentration, the curve photographed represents protein concentration gradient.

Theoretical

The developments described here were suggested initially by observations on the rabbit papilloma virus protein (4) and, subsequently, by others on the pseudoglobulin GI of horse serum (5). In an attempt to find a means for characterizing the Svensson curves obtained with these proteins, there was found an apparent close relation between the standard deviation of these curves and the time of boundary migration in unit electric field so that

$$\frac{\Delta\sigma}{E\Delta t} \cong H \text{ (constant)}$$

Here $\Delta\sigma$ is the change in standard deviation of gradient curves taking place in the corresponding time Δt at the field strength E. H is a value determinable by experiment. Under the conditions of the experiments and with the observed findings, the application of H computations seemed a useful method for curve characterization. However, the nature of the data obtained in this way indicated the possibility of a fundamental significance of the H value. This has been supported by the following theoretical considerations.

Let us examine the behavior of a boundary between pure buffer and the same buffer solution in which the concentration of monodisperse protein is C. If it is assumed that no discontinuity in field strength or pH occurs at this boundary, the application of an electric field will cause it to move as a whole with the mean velocity $\bar{V}$ in the x direction, so that $\bar{V} = d\bar{x}/dt$ where $\bar{x}$ is the abscissa of the centroid of area under the Svensson gradient curve. These curves may be expressed as follows:

$$\frac{\partial n}{\partial x} = K\frac{\partial C}{\partial x} = f(x, U, t) \tag{1}$$

where n is the refractive index, t the time of migration, U the mobility of the individual ion, and K the specific refractive index increment for the protein.

Before attempting to set up a general expression for the function $f(x, U, t)$ to describe these gradient curves, we may make some simplifying assumptions which will be of practical value. The definition of mobility, U, of a protein ion is as follows:

$$U = V/E \tag{2}$$

where V is the observed velocity and E the electric field. If all the monodisperse protein ions have the same mobility,[2] the spreading of this boundary is due only to diffusion and other irreversible causes. The latter being neglected for the present, the refractive index will then vary across the boundary according to the equation

$$\frac{\partial n}{\partial x} = \frac{n_1 - n_2}{2\sqrt{\pi D_1 t}} e^{-(x-UEt)^2/4D_1t} \tag{3}$$

where n_1 and n_2 are the refractive indices of the protein solution and buffer, respectively, and where it has been assumed that the boundary was perfectly sharp at $t = 0$.

In this equation D_1 is a constant analogous to the diffusion coefficient but not necessarily the same, since variations in mobility of individual particles with time may affect the rate of diffusion under the influence of an electric field. Equation 3 then describes a boundary moving with velocity $V = UE$ in the x direction and showing diffusion according to the modified diffusion constant D_1 but no reversible spreading. These conditions have not yet been attained experimentally; however, the equation has an interesting special case. When $E = 0$, one has a diffusion experiment of the usual type and Equation 3 reduces to the one proposed by Wiener (6)

$$\frac{\partial n}{\partial x} = \frac{n_1 - n_2}{2\sqrt{\pi D t}} e^{-x^2/4Dt} \tag{4}$$

In this case D is the true diffusion constant. It should be observed that this boundary gradient curve is the same as the normal error curve and that D can be calculated very simply from the second moment of the curve

$$D = \sigma^2/2t \tag{5}$$

[2] In case there are fluctuations in charge which make the mobility fluctuate in time, it is sufficient that the time-average mobility is the same for all ions.

where σ^2 is the second moment (σ is the standard deviation) and t the time of diffusion.

If instead of one mobility the protein ions have a distribution of mobilities $g(U)$ so that $g(U)dU$ is the fraction of the ions with mobilities between U and $U + dU$, the equation becomes

$$\frac{\partial n}{\partial x} = \frac{n_1 - n_2}{2\sqrt{\pi D_1 t}} \int_{-\infty}^{\infty} g(U) e^{-(x-UEt)^2/4D_1t}\, dU \tag{6}$$

This expression describes a curve which progresses at the average rate $\overline{V} = \overline{U}E$. The moving ions are subject to modified diffusion (D_1 of Equation 3), but the boundary is now further spread by the distribution in mobility among the particles.

The quantity actually measured in Equation 6 is $\partial n/\partial x$, which is given by curves of the kind shown in Figs. 1, 3, and 5. From these curves it is desired to find the distribution in mobilities, $g(U)$, which will be characteristic of the protein under analysis. This can be done easily in one special case; namely, when the spreading due to diffusion is small[3] compared with the spreading due to the distribution in mobility. In this case the exponential factor in Equation 6 is a sharply peaked function of U and the only contribution to the integral comes from the vicinity of this peak. Here $g(U)$ can be considered to be constant with its value $g(x/Et)$ and can be taken outside the sign of integration. We then have

$$\frac{\partial n}{\partial x} = \frac{n_1 - n_2}{Et}\, g(x/Et) \tag{7}$$

which gives the relationship between the gradient ($\partial n/\partial x$) curve photographed and the mobility distribution sought.

Perhaps the simplest experimental test of the validity of these assumptions is found in the consequence of Equation 7 that the

[3] In case diffusion cannot be neglected, it is still possible to find a solution for $g(U)$ from Equation 6. This takes the form of a modified Laplace integral and is

$$g(U) = \frac{E}{(n_1 - n_2)2i} \sqrt{\frac{t}{\pi D_1}} \int_{-i\infty}^{i\infty} \frac{\partial n}{\partial x} e^{(x-UEt)^2/4D_1t}\, dx$$

This integral may be conveniently evaluated if diffusion is not too large by the method of steepest descents.

standard deviation of the gradient curve is proportional to time. For by definition

$$\sigma^2 = \frac{\int \frac{\partial n}{\partial x}(x - \bar{x})^2\,dx}{\int \frac{\partial n}{\partial x}\,dx} = \frac{n_1 - n_2}{Et}\,\frac{\int g(x/Et)(x - \bar{x})^2\,dx}{n_1 - n_2}.$$

When $U = x/Et$ and $\bar{U} = \bar{x}/Et$ are substituted,

$$\sigma^2 = (Et)^2 \int_{-\infty}^{\infty} g(U)(U - \bar{U})^2\,dU \tag{8}$$

Here the integral, which is the second moment of the mobility distribution curve, is independent of time. If this integral is designated as H^2, there follows the relationship

$$\frac{\Delta\sigma}{E\Delta t} = H \text{ (constant)} \tag{9}$$

This is identical with the relation derived empirically from experimental data with the papilloma protein and the pseudoglobulin GI.

DISCUSSION

The expressions derived indicate that, under proper conditions, spreading of migrating boundaries can be explained on the basis of a distribution of mobilities among the protein ions. The validity of some of the assumptions in the development and the limits of interpretation based on the expressions for any given material, however, are dependent on the conditions and results of actual experiments with the particular material. Analyses from this point of view have been made with data obtained with the two monodisperse protein systems already mentioned, the pseudoglobulin GI from horse serum (5) and the rabbit papilloma virus protein (4). The results of certain of these examinations have been described in the separate papers cited. These will be summarized briefly, and in addition, other results, not reported before, will be included.

It is recognized that many factors may contribute to boundary spread of a monodisperse protein during electrophoresis. Grouped into categories with respect to the criteria applicable for experi-

mental test, the principal factors are considered to be (1) properties of the protein ions including mobility and its variation among the population studied as well as shape and size of the ions, (2)

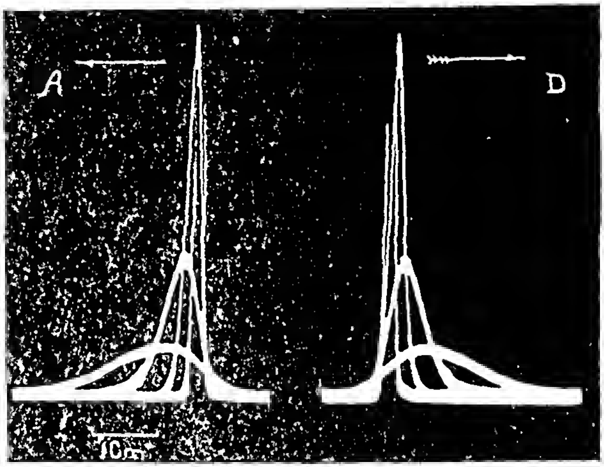

FIG. 1. Svensson diagrams of pseudoglobulin GI migrating in 0.1 ionic strength buffer at pH 7.6. $E = 4.67$ volts per cm. *D* denotes the descending boundary migrating into protein and *A* the ascending boundary migrating into buffer.

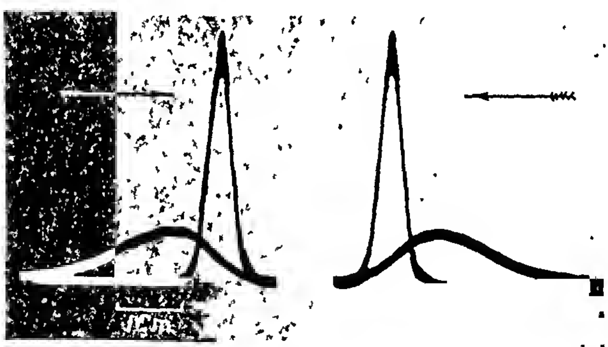

FIG. 2. Svensson diagrams showing regained boundary sharpness on reversal of the electric field in the experiment of Fig. 1. The first curve is identical with the last curve of Fig. 1. The time of return migration was 16,080 seconds.

diffusion, (3) convection due to temperature gradients set up in the cell by the Joule effect, and (4) gradients at the boundary other than those of protein concentration.

The contributions of these factors to the findings in experiments

with pseudoglobulin GI and the papilloma virus protein may now be considered in reverse order of the statement above. Gradients in pH and electric field are sometimes present at the boundary, causing reversible boundary spread and other anomalous behavior. Longsworth, Cannan, and MacInnes (7) are of the opinion that, when such disturbances have been reduced to a negligible value,

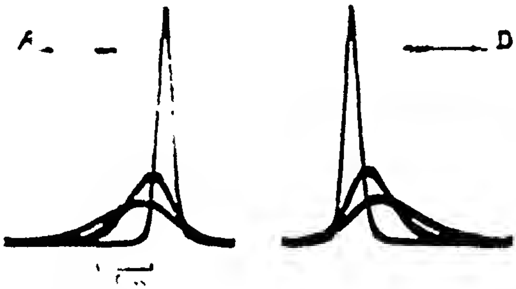

FIG. 3. Svensson diagrams of pseudoglobulin GI reversibly denatured from 5 M urea migrating in 0.1 ionic strength buffer at pH 7.6. $E = 3.62$ volts per cm. *D* denotes the descending boundary migrating into protein and *A* denotes the ascending boundary migrating into buffer.

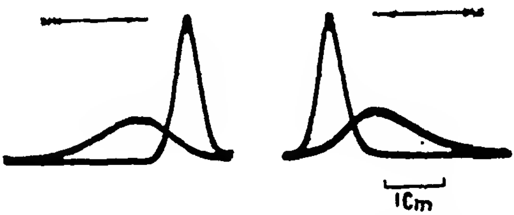

FIG. 4. Svensson diagrams showing regained boundary sharpness on reversal of the electric field in the experiment of Fig. 3. The first curve is identical with the last curve of Fig. 3. The time of return migration was 21,040 seconds.

the ascending and descending boundaries have the same shape. In Fig. 1, which is a reproduction of Fig. 3 of the preceding paper (5), are successive Svensson curves of both ascending and descending boundaries of pseudoglobulin GI at pH 7.6 in 0.1 ionic strength buffer. Each boundary is single and no evidence is seen of electroendosmotic effects. The two sets of curves representing ascending and descending boundaries appear to be mirror images as

judged by contour. In previous work (4) and above, the use of the H value of Equation 9 has been suggested as a measure of boundary characters. Application of this criterion in the present

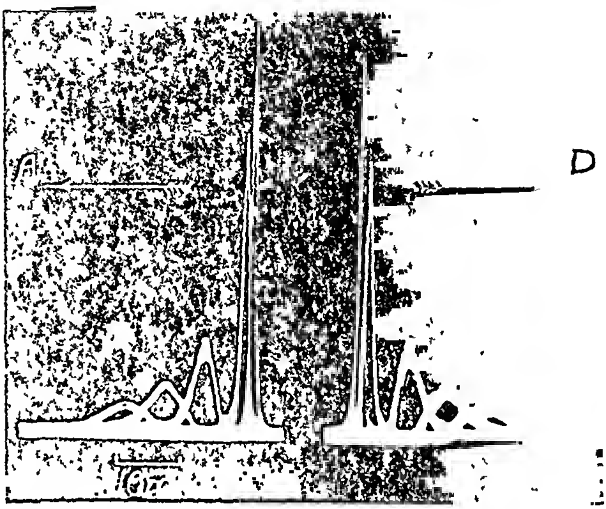

Fig. 5. Svensson diagrams of rabbit papilloma virus protein migrating in 0.1 ionic strength buffer at pH 6.54. E = 2.60 volts per cm. D denotes the descending boundary migrating into protein and A the ascending boundary migrating into buffer.

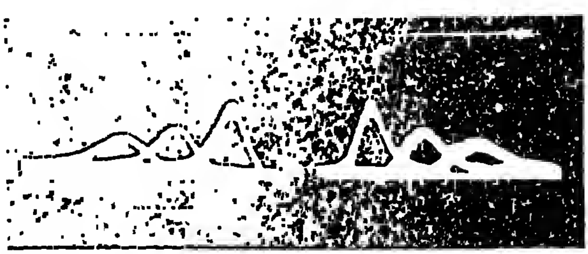

Fig. 6. Svensson diagrams showing regained boundary sharpness on reversal of the electric field in the experiment of Fig. 5. The first curve is identical with the last curve of Fig. 5. The time of return migration was 78,300 seconds.

experiment has yielded the values given in Table I. These were obtained by calculation of H for successive pairs of curves of the ascending and descending boundaries, respectively, of Fig. 1. One pair of curves not appearing in Fig. 1, but used in Table I, was photographed between the third and last pair shown in Fig. 1.

From Table I it is evident (1) that the successive H values for both boundaries show no systematic variation and (2) that the average value for the ascending curves is similar to that obtained for the descending curves. It thus seems that the curves of the ascending boundary are close mirror images of the corresponding ones of the descending boundary not only in form, as judged by inspection, but also in rate of spread, as estimated from similarity of the H values. Consequently, in so far as the existence of mirror images is an acceptable criterion, it may be considered that gradients at the boundary other than protein concentration were of

TABLE I

Statistics and Values of Heterogeneity Constant H for Svensson Curves with Pseudoglobulin GI in Experiment of Fig. 1

Curve No.	Time	Descending boundary			Ascending boundary		
		s	Δs	$H \times 10^6$	s	Δs	$H \times 10^6$
	sec.	cm.	cm.	sq. cm. sec.$^{-1}$ volt^{-1}	cm.	cm.	sq. cm. sec.$^{-1}$ volt^{-1}
1		0.110			0.110		
	4920		0.155	6.86		0 151	6.82
2		0.265			0.273		
	6180		0.215	7.57		0.228	8 03
3		0.480			0.501		
	5820		0.178	6.66		0.216	8.08
4		0.658			0.717		
Mean				7.05			7.70

negligible influence on spread in this experiment. Similar findings obtained in comparison of the two boundaries in other experiments with pseudoglobulin GI in the pH range between 5.5 and 7.6 have been shown in Table I of the preceding paper (5). Examinations of pseudoglobulin GI reversibly denatured from urea (5) have revealed mirror images for this material also, as shown in Fig. 3 ((5) Fig. 4).

The influence of diffusion and other causes of irreversible spread has been examined by measurement of the reversibility of boundary spread in the instances both of the native pseudoglobulin GI and of the material reversibly denatured from urea. An example of the findings with the native protein is shown in Figs. 1 and 2

and in Table II. In Fig. 1 are the successive curves seen in forward migration of both boundaries. When the last curve had been photographed, the current was reversed and, during return migration, the diagram of Fig. 2 was obtained. It is seen that the degree of reversibility was apparently great.

TABLE II

Mobility, U; Heterogeneity Value, H; Modified Diffusion Coefficient, D_1 of Equation 6; and Diffusion Constant of Pseudoglobulin GI and Papilloma Virus Protein Observed on Forward Migration and on Return Movement after Current Reversal

Material	Boundary*	$U \times 10^5$	$H \times 10^6$	$D_1 \times 10^7$ (25°)	$D \times 10^7$ (25°)
		sq. cm. sec.$^{-1}$ volt^{-1}	sq. cm. sec.$^{-1}$ volt^{-1}		
Pseudoglobulin GI from horse serum, pH 7.6, 0.1 ionic strength buffer	*D*	−0.98	7.05	4.18	4.43
	A	−1.13	7.70	5.29	
Pseudoglobulin GI reversibly denatured from 5 M urea, pH 7.6, 0.1 ionic strength buffer	*D*	−0.78	6.10	6.17	†
	A	−0.84	6.18	6.58	
Rabbit papilloma virus protein, pH 6.54, 0.1 ionic strength buffer	*D*	−0.94	1.44	6.38	0.665
	A	−0.91	1.46	7.64	

* *D* denotes descending boundary migrating into protein and *A* ascending boundary migrating into buffer.

† The value *D* for the pseudoglobulin GI reversibly denatured from 5 M urea has not been given but has been reported (5) as similar to that of the native material given here.

The modified diffusion constants were calculated (Equation 3) for these experiments as follows:

$$D_1 = \frac{\Delta\sigma^2}{2t_2} = \frac{\sigma_3^2 - \sigma_1^2}{2t_2} \tag{10}$$

where $\Delta\sigma^2$ is the difference between the second moment, σ_1^2, of the initial starting curve (Fig. 1) and σ_3^2 of the final reversal curve (Fig. 2) seen in the time interval, t_2, and D_1 the modified diffusion coefficient defined in Equation 6. The values of D_1 obtained for the native protein and for that reversibly denatured from urea, respectively, and corrected for temperature 25°, are shown in

Table II. It is of interest to compare also the average D_1 of the ascending and descending boundaries with the diffusion constant D (5). This value, D_1, for the native protein is very close to that of D. In the instance of the protein reversibly denatured from urea, D_1 was larger than D (5).

From these calculations of D_1 information regarding the nature of the observed irreversible spread is obtained. In the experiment of Fig. 1, the similarity of D_1 to D indicates that the irreversible spread was due to diffusion. In that of Fig. 3, D_1 was larger than D, suggesting that here some additional irreversible spread occurs.

For application of the theoretical considerations to the papilloma virus protein, seven experiments made as previously described (4) were available for analyses similar to those made for the pseudoglobulin GI discussed above. Due to the very considerable difficulty in obtaining this animal virus protein in amounts large enough for this sort of work, the data are fewer than those with pseudoglobulin GI. An additional impediment was the difficulty in obtaining the protein in concentration optimum for observation without excessive opalescence. The interpretations, therefore, are made with consideration of these facts.

In Fig. 5 are Svensson curves obtained with the papilloma protein at pH 6.54 in 0.1 ionic strength buffer solution. Diagrams at other pH values have already been shown (4). As in the instance of pseudoglobulin GI, the curves of the ascending boundary appear to be close mirror images of those of the descending boundary. The values of H in this experiment and others are given in Table III. The data are not sufficient to provide unequivocal evidence either of random or systematic variation in the values of H for successive curves of the ascending and descending boundaries of a given experiment. On the other hand, it is evident that within the limits of the data, the variation, though considerable in individual instances, is relatively small when the results are viewed as a whole. It is observed that H values for the ascending boundaries are quite similar to those of the corresponding descending boundaries in the various experiments. Further, there are definite indications of a trend toward progressive increase in the average H values with increase in pH ((4) Fig. 3). Though it is not possible from these limited data to state that H was actually constant in the instance of the papilloma protein, it is clear,

nevertheless, that the curves of corresponding pairs of boundaries are essentially mirror images and that, within the limits of the

TABLE III

Statistics and H Values for Svensson Curves with Papilloma Virus Protein

Protein sample	pH	Curve No.	Time	Descending boundary			Ascending boundary		
				σ	$\Delta\sigma$	$H \times 10^6$	σ	$\Delta\sigma$	$H \times 10^6$
			sec.	*cm.*	*cm.*	*sq. cm. sec.$^{-1}$ volt^{-1}*	*cm.*	*cm.*	*sq. cm. sec.$^{-1}$ volt^{-1}*
B	3.78	1		0.0918			0.0884		
			6,600		0.0098	0.46		0.0168	0.78
		2		0.1016			0.1052		
			6,600		0.0222	1.03		0.0151	0.70
		3		0.1238			0.1203		
Mean ..						0.74			0.74
A	3.81	1		0.0643			0.0753		
			8,400		0.0130	0.34		0.0195	0.51
		2		0 0773			0.0948		
			8,400		0 0373	0 97		0.0137	0.36
		3		0.1146			0.1085		
Mean ..						0.65			0.43
A*	6.54	1		0.0601			0.0534		
			25,200		0.1080	1 65		0.1223	1.87
		2		0.1681			0.1757		
			25,200		0.0885	1 35		0.1006	1.54
		3		0 2566			0.2763		
			25,200		0.0873	1 33		0.0634	0.97
		4		0.3439			0.3397		
Mean .						1.44			1.46
B	6 56	1		0.0868			0.0845		
			25,260		0.0555	0.92		0.0484	0.80
		2		0.1423			0.1329		
			10,920		0.1618	1.65		0.1311	1.34
		3		0.3041			0.2640		
Mean						1 28			1.07

* Experiment of Fig. 5.

variations observed, pH and electric field gradients exerted little effect on the spread of boundary in the pH regions studied.

The results of studies on current reversal with the papilloma

protein are illustrated in Fig. 6 which shows the return curves with the preparation of Fig. 5. From inspection the final return curves give evidence of reversal of spread, though the sharpness regained is much less than that of the initial curves of Fig. 5. The corresponding values of D and D_1 are shown in Table II. The modified diffusion constant, D_1, was of a different order from that of D, the previously reported (8) diffusion constant of this protein. From the value of D_1 it would appear that by far the greater part of the irreversible spread was due to causes other than true diffusion.

The findings with pseudoglobulin GI, both native and reversibly denatured from urea, in the pH range between 5.5 and 7.6 have shown clearly that boundary spread during its electrophoretic migration is due only in minor degree, if at all, to certain of the factors enumerated above. There is left for consideration only such reversible boundary spread as that which could arise from a distribution of mobilities among the ions. Evidence (5) has been reported for the monodispersity of the protein used in analyses and, furthermore, it has been suggested (9) that, under suitable experimental conditions, small differences in the size of the ions should contribute negligibly to mobility differences and to the reversible spread of the migrating boundary. It seems reasonable, then, to conclude that in the pH region between 5.5 and 7.6, where H is constant, boundary spread of this protein is dependent on a distribution of mobilities among the protein ions of the preparations studied. This distribution is given by Equation 7 and its standard deviation has been shown to be H of Equation 9. Such mobility distribution might in turn be translated into a corresponding surface charge density distribution by proper application of the Debye-Hückel-Henry theory (9).

In the instance of the papilloma virus protein, there was substantial evidence that gradients of pH and electric field at the boundary were probably of negligible influence on boundary spread. On reversal of the current, a large percentage of the boundary spread occurring during forward migration was lost in the return movement. Much of the spread, however, was irreversible and the factors responsible for this finding are not clear from the present work. The diffusion constant of the material is small (8) and, consequently, diffusion would not be expected to play an important rôle. Irreversible spread other than that

associated with diffusion may have been due to convection. On the other hand, it is possible that the rate of diffusion of the protein ions moving in an electric field is altered by fluctuations in ion charge and mobility as discussed in connection with Equation 3. Such fluctuations would lead to added irreversible spread in the presence of the electric field.

In the case of pseudoglobulin GI, H has been considered a constant, based both on the immediate values of H and on the validity of the assumptions in its derivation as indicated by experiment. For this reason and because of its significance with respect to the nature of the material, H has been designated as the heterogeneity constant (5) for pseudoglobulin GI. For the papilloma protein the significance of calculated values of H is not the same as that of the values for pseudoglobulin GI. In order that H be constant it is necessary that the standard deviation of the Svensson curve be directly proportional to time of migration. Thus diffusion must be either absent or present to such a small extent that its influence is not detected by the experimental method of measuring. These conditions held for pseudoglobulin GI where the similarity of D_1 and D indicated that the small degree of irreversible spread experienced was essentially all diffusion. For the papilloma protein, however, there was a great difference between the true diffusion constant, D, and the modified constant, D_1. The reasons for this are not clear, and the data are not complete enough to determine whether or not σ was actually proportional to time. On the other hand, the data do not indicate any great departure of H from constancy. For the present, H for the papilloma protein has been designated as a value (4) determinable by experiment. Nevertheless, H may be considered descriptive of boundary characters, furnishing an estimate of rate of boundary spread due to factors concerned with both diffusion and electrophoresis. The magnitude of H in all cases is an index or coefficient of total boundary spread and as such establishes a limit beyond which observed spread cannot be due to heterogeneity of particle mobility. On this basis, for instance, it appears that the papilloma protein exhibits a greater degree of electrophoretic homogeneity than the pseudoglobulin GI, though the observed values of H for the former are interpretable only qualitatively.

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SUMMARY

A general equation has been set up to express the dependence of refractive index gradient on time, distance moved, and individual ionic mobility in the moving boundary of a monodisperse protein seen in electrophoresis experiments. This relationship takes into account the effects of diffusion and a distribution of mobilities among the protein ions in reversible boundary spread.

In cases of low rate of diffusion or wide distribution in mobilities (heterogeneity) the general equation is greatly simplified and, at a constant electric field strength, the standard deviation of observed Svensson curves increases at a constant rate with time. This rate of increase is then itself, except for a constant factor, the standard deviation of the mobility distribution function. Absolute values of the mobilities in this distribution may be estimated by means of existing theory.

Data have been presented in the form of Svensson curves for test of the validity of the assumptions and development of the relations described in the instances of pseudoglobulin GI of horse serum and the rabbit papilloma virus protein.

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SPECTROPHOTOMETRIC DETERMINATION OF IRON

I. USE OF MERCAPTOACETIC ACID

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It is the purpose of the research reported in the present series of papers to establish accurate spectrophotometric procedures for the determination of iron, more particularly for use in biochemical and nutrition studies, but applicable generally to the analysis of biological materials, foods, pharmaceuticals, and chemical reagents. Several colorimetric methods for the determination of iron are available, but they lack the rapidity and precision of spectrophotometric methods. Colorimetric procedures are not directly applicable to spectrophotometric work, and are apt under spectrophotometric study to show serious flaws and limitations. In fact, the interferences commonly met in analyses of foods and biological materials make it doubtful whether an investigator desiring accurate results in iron determinations should be satisfied until checks are obtained by at least two different methods.

For this reason the present research was designed to adapt for spectrophotometric use a number of the best colorimetric methods for iron, to obtain accurate calibration data over a wide range of iron concentrations, and to establish the validity of these data for determining iron in solutions containing common acids in concentrations that occur in analytical solutions prepared by various wet and dry methods of ashing. This information could be used in testing methods for ashing samples, in studying the effect of interfering substances other than the acids introduced in preparing the analytical solutions, and in devising rapid and accurate methods for determining total iron in foods and biological materials. It also seemed that such work as that outlined was a prerequisite to any successful attack on the problem of developing

trustworthy analytical techniques for determining biologically available iron.

One of the reagents selected for study was mercaptoacetic (thioglycolic) acid. Swank and Mellon (1) have listed applications of this reagent, and describe experiments showing its high degree of freedom from interferences.

Apparatus and Reagents—

A Coleman model 10-S-30 spectrophotometer was used to measure transmittances. Matched square cuvettes were used to hold reference liquids and test solutions. The dark current adjustment was frequently checked during the measurements, which were made between 29–31°.

15 N nitric acid, 6 N hydrochloric acid, 9 N perchloric acid, 36 N sulfuric acid, trichloroacetic acid, and ammonium hydroxide were purified by distillation methods. 10 N iron-free sodium hydroxide was obtained by electrolysis in platinum at 5 amperes for 30 hours, with a rotating cathode. The reagent (ammonium mercaptoacetate) was made by adding 40 ml. of mercaptoacetic acid to 300 ml. of 4 N ammonium hydroxide and diluting to 500 ml. with distilled water. Three primary standard solutions containing 0.2000 mg. of iron per ml. were used in making all more dilute standard and test solutions. One primary standard was made by dissolving 1.405 gm. of ferrous ammonium sulfate hexahydrate (Mohr's salt) in 200 ml. of water, adding 10 ml. of 36 N sulfuric acid and 20 ml. of saturated bromine water, boiling off excess bromine, and making to a liter. Another was made by dissolving 0.4000 gm. of pure iron wire in 10 ml. of 36 N sulfuric acid, 10 ml. of 6 N hydrochloric acid, and 4 ml. of 15 N nitric acid, heating to remove the excess of volatile acids, and making to 2 liters. A third solution contained 1.727 gm. of recrystallized hydrated ferric ammonium sulfate (mol. wt. 482.2) and 5 ml. of 36 N sulfuric acid per liter. Transmittance readings with three sets of test solutions made from these standards and compared spectrophotometrically at appropriate wave-lengths, with use of mercaptoacetate, α,α'-dipyridyl, and ferron reagents, showed that all three standards had the same iron content, within 1 part in 400. Additional checks were obtained by gravimetric, volumetric, and electrometric methods, but the spectrophotometric check was the most accurate and convenient.

Summary of Calibration Experiments

Calibrations were made by the procedure given below for analyses, except that the test solutions contained known quantities of iron; concentrations of acids, mercaptoacetate reagent, and ammonium hydroxide were varied systematically to find permissible, adequate, or optimum quantities, and the systems were kept under observation for at least 12 hours. Different combinations of primary standards and reagents were used to eliminate constant errors.

The median transmittances obtained in about 400 observations made with 98 test solutions are recorded in Table I. These medians were calculated from the readings taken about 30 minutes

TABLE I

Concentration-Transmittance Data for Iron Determinations

Fe per 50 ml.	Transmittance		Average deviation of single observations
	Blank reference	Water reference	
mg.	*per cent*	*per cent*	*per cent*
0.0500	81.0	80.3	0.2
0.1000	66.2	65.6	0.3
0.200	43.6	43.2	0.3
0.300	29.2	28.8	0.4
0.400	18.9	18.7	0.7
0.500	12.7	12.5	1.1
0.600	(9.0)	(8.5)	0.9

after color development. However, transmittances read any time between 5 minutes and 12 hours after color development generally agreed within 0.4 per cent; only a few slightly high values were obtained in the 5 minute readings. Solutions stored in cuvettes showed some tendency to fade. This was not observed in solutions stored in glass-stoppered Pyrex Erlenmeyer flasks for 30 minutes; after longer standing it is advisable to shake the solutions a little and allow air bubbles to disappear before the readings are taken.

The reagent made with mercaptoacetic acid purified by fractional distillation in a vacuum gave results identical with that made from Eastman's practical reagent. When only 5 ml. of mercaptoacetate reagent were used per 50 ml.—about twice the

amount used in the colorimetric procedure of Leavell and Ellis (2)—Beer's law was not followed at high iron concentrations, and the colors faded badly on standing and could not be restored by shaking. 10 ml. quantities of the reagent were used in obtaining the data in Table I, which show on plotting that Beer's law is followed exactly. Instrumental errors make it undesirable to use transmittances lower than 10 per cent in analyses.

Transmittances for systems without added acid agreed closely enough with those for systems with added acid to show that acids in concentrations up to those specified in the procedure below have no detectable effect on the color. Addition of 0.5 ml. of 30 per cent hydrogen peroxide per 50 ml. did not interfere with the color development, but when 1.0 ml. was added no color appeared.

Study of the data indicated an optimum pH between 9.2 and 9.5, but the excess of 4 N ammonium hydroxide may vary between 1 and 4 ml. without causing transmittance changes exceeding 0.5 per cent. Sodium hydroxide was substituted for ammonium hydroxide in about 5 per cent of the test solutions, and gave transmittances in excellent agreement with the medians in Table I.

Spectrophotometric Procedure for Total Iron

The following procedure is based on the experiments described above, and has been further tested by comparison with similar procedures with the reagents α,α'-dipyridyl and ferron, in analyses of some 80 materials, including foods and food concentrates, beverages, urine, feces, and pharmaceuticals.

Procedure—Ash a measured sample and make it up to a suitable volume after hydrolyzing any pyrophosphate present (3, 4). Take for analysis an aliquot containing not more than 0.55 mg. of iron. If a wet ashing method was used, evaporate the aliquot to dryness and destroy organic matter by heating the residue in succession with 0.5 ml. portions of 36 N sulfuric acid and 30 per cent hydrogen peroxide, or by electrical heating in deep fused silica beakers, with precautions to avoid loss of iron. Take up the residue with 5 ml. of 6 N hydrochloric acid and 0.3 ml. of 15 N nitric acid, dilute to 20 ml., and reflux for 30 minutes to hydrolyze pyrophosphate.

In all cases treat the aliquot to remove any interfering inorganic substances known to be present (1, 5) if maximum accuracy is desired. The solution for analysis may contain up to 5 ml. of 6 N

hydrochloric acid, 1 ml. of 36 N sulfuric acid, 5 ml. of 9 N perchloric acid, 2 ml. of 15 N nitric acid, 20 ml. of 0.6 N trichloroacetic acid, or 2 ml. each of 9 N perchloric and 15 N nitric acid.

If necessary, evaporate the solution to a volume which allows for reagent additions. Add a small piece of Congo red paper and neutralize with 4 N ammonium hydroxide. Add 10 ml. of mercaptoacetate reagent, then 2 ml. of 4 N ammonium hydroxide, and make to exactly 50 ml. Filter through a dry Pyrex crucible before diluting to volume if a precipitate forms. Make duplicate transmittance readings on two portions of the clear solution in about 30 minutes, at a wave-length of 535 mμ. Calculate the result of the analysis by substituting the median value of T in the proper equation, as indicated below, or by use of a graph derived from the equation.

If the reagents are free from iron use water as the reference liquid; otherwise use a blank containing the reagents. If a colored aliquot is used directly, prepare as a reference liquid a system containing no mercaptoacetate reagent and the same volumes of colored liquid and ammonium hydroxide as the test solution.

For convenience in calculating results with maximum precision the data in Table I have been reduced by the method of least squares to two equations. In the first,

$$\text{Mg. Fe per 50 ml. test solution} = \frac{-0.731 \log_{10} T + 1.460}{l} \qquad (1)$$

T is the percentage transmittance relative to the blank, and l is the thickness of the solution in cm. With water as the reference liquid the constants are respectively −0.727 and 1.451. In the present calibration l was 1.308 cm. The actual value of l should be determined for the particular cuvettes used, with a micrometer and calipers. Under the conditions specified the average deviations of T for various iron concentrations are about half as large as those given in Table I for single observations.

SUMMARY

An accurate spectrophotometric method for the determination of iron with mercaptoacetic acid has been developed experimentally.

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CARBOHYDRATE METABOLISM IN THIAMINE DEFICIENCY*

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The relationship of vitamin B_1 to carbohydrate metabolism was one of the earliest functions of a vitamin to be observed. The problem has been more or less continuously investigated up to the present time and the majority of the evidence indicates that in vitamin B_1 deficiency there is some interference with glycogen storage, hyperglycemia, and characteristic errors in carbohydrate metabolism. Ever since the discovery of the rôle of thiamine as a constituent of cocarboxylase (1) and its consequent effect on pyruvate oxidation (2), studies *in vitro* of cellular metabolism in thiamine deficiency have indicated that a rather generalized disturbance of carbohydrate metabolism exists. Thus Meiklejohn, Passmore, and Peters had reported that the brain of avitaminotic pigeons exhibited a lower oxygen uptake in the presence of lactate when compared to normal brain tissue (3). Barron and Lyman (4) in a study of the ability of kidney slices to synthesize carbohydrate in the presence of pyruvate reported that tissues from vitamin B_1-deficient rats were unable to accomplish this transformation when compared to normal controls. Similar experiments by Lipschitz, Potter, and Elvehjem (5) revealed that liver and kidney tissue from vitamin B_1-deficient pigeons showed a diminished ability to remove pyruvate which was restored in the liver tissue when glucose was administered to the polyneuritic birds.

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The present investigation was designed to study certain aspects of carbohydrate metabolism in animals suffering from an inadequate intake of thiamine. Since such a study is complicated by effects due to inanition or a moribund state, a subacute deficiency was produced by the administration of suboptimal amounts of thiamine. This deficiency state was characterized by a relatively constant weight and food intake but a pronounced disturbance in intermediary metabolism of carbohydrate as evidenced by studies of the urinary pyruvate according to the procedure of Harper and Deuel (6).

Under these conditions studies of the rate of absorption of glucose from the intestine and the rate and extent of hepatic glycogenesis and glycogenolysis correlated with the blood sugar level were carried out. In addition, a study of the ability of the avitaminotic organism to convert ingested lactate and pyruvate into glycogen was undertaken. Finally, confirmatory evidence of the phenomena observed in connection with glycogen metabolism in the liver was obtained by a study of the ketolytic action of glucose in thiamine deficiency.

EXPERIMENTAL

Rats, 120 to 160 gm. in weight, were selected from our stock colony for these experiments. Males were used for all of the absorption and glycogen investigations and females for the ketosis studies. The experimental plan involved a comparison of animals maintained on a diet deficient in thiamine with those adequately nourished with respect to this factor. Such normal animals were always maintained on the deficient diet supplemented with 100 γ each of thiamine, riboflavin, and pyridoxine per week,[1] for several days prior to the experiment. The depletion of the thiamine reserves and maintenance of the subacute avitaminotic state were accomplished by the use of a diet formulated and supplemented as previously described (6). The adequacy of this diet so supplemented was assayed by preliminary studies of its growth-promoting power. These observations indicated that only thiamine was the limiting factor, for the growth rate was proportionate to the degree of thiamine supplementation.

[1] Crystalline supplements were obtained from Merck and Company, Inc.

Absorption of Glucose from Intestine—Preparatory to studies of the rate and extent of glycogenesis, the effect of a deficiency in thiamine on the rate of glucose absorption from the intestine was studied. For the determination of the coefficient of absorption of glucose the procedure of Cori (7) was used. Following a 24 hour fast during which only water was allowed, the animals of the various experimental groups were fed glucose by stomach tube. Absorption was allowed to proceed for 1 or 2 hours, when the animal was anesthetized by the intraperitoneal injection of sodium amytal. The entire intestinal tract from duodenum to rectum was then dissected and the contents washed out by means of warm water administered by stomach tube. Further treatment and assay of the glucose in the gut washings was carried out as described by Deuel *et al.* (8). The glucose recovered has been corrected to conform to the limitations of the technical procedure which proved to be capable of accounting for 95 per cent of the administered glucose in control experiments in which lavage was completed as soon as possible after oral administration of the sugar.

The estimation of absorption rates at the end of 1 hour is not reliable because of marked differences in the rate of gastric motility. Since little or no glucose is absorbed from the stomach (9, 10), absorption cannot take place until the sugar enters the intestine. One may thus observe markedly varying absorption rates during this period (11). For this reason a statistical appraisal of the apparent differences in the rate of absorption by the Fisher t method (12) fails to prove them significant. The 2 hour period of comparison is more valuable and in these experiments it has been used to determine the absorption rates for glucose.

In the experiments reported in Table I it is apparent that a significant decrease in the rate of absorption of glucose has been observed, amounting to 27 mg. or 17 per cent of normal. This relatively mild but definitely decreased absorption would appear to substantiate further the belief that in this case one is dealing with a controlled subacute deficiency state. In view of the well known observation that diminished function of the alimentary tract is a common attribute of thiamine deficiency (13–17), this quantitative evidence for retarded glucose absorption is not surprising. Whether this effect is due to the atony of the gastro-

intestinal tract or the thiamine deficiency *per se* has not been proved by these experiments.

Glycogenesis, Glycogenolysis, and Blood Sugar Levels—Male rats, normal or thiamine-deficient, were fasted 48 hours with water *ad libitum* allowed during this period. Certain of the animals were then anesthetized by the intraperitoneal injection of sodium amytal and the liver glycogen and blood sugar determined to obtain control values for these factors. Others were fed glucose by stomach tube, 1 cc. of a 35 per cent solution per 100 gm. of body weight, and

TABLE I

Comparison of Glucose Absorption in Normal and Thiamine-Deficient Male Rats

Period of absorption	Thiamine status	No. of experiments	Average weight	Glucose fed	Glucose recovered	Coefficient of absorption*	Difference	t calculated	Significant value for t†
hrs.			*gm.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm. per hr.*	*mg. per 100 gm. per hr.*		
1	Normal	10	132	335	152	183.0 ± 14.4			
	Deficient	11	122	320	159	161.0 ± 9.2	22	1.24	2.86
2	Normal	10	139	337	19	159.0 ± 3.3			
	Deficient	10	124	326	62	132.0 ± 3.1	27	5.66	2.88

* Including the standard error of the mean calculated as follows: $\frac{\sqrt{\Sigma d^2/n}}{\sqrt{n}}$

† Based on a P value of 0.01 (chances of difference in result being due to errors in sampling 1 in 100).

sacrificed 3, 6, or 12 hours after the feeding in order to determine the liver glycogen and blood sugar at these various intervals.

The determination of the blood sugar was made by removal of blood directly from the ventricle exposed after anesthesia had supervened. The method of Folin and Malmros (18) was used for assay of the blood samples, with colorimetric comparison carried out by means of a Klett-Summerson photoelectric colorimeter having the No. 54 filter. Immediately after the blood samples were obtained, the liver was dissected out and glycogen determined by the usual procedure of this laboratory (19). The results of these experiments are detailed in Table II.

A definitely diminished quantity of glycogen was found in the liver of the thiamine-deficient rats observed 3 or 6 hours after the feeding of glucose when a comparison with similarly treated normal rats was made. The diminished absorption of glucose in the

TABLE II

Liver Glycogen and Blood Sugar after Glucose

Period after feeding	No. of experiments		Thiamine status*	Average liver glycogen†	Difference	t calculated	Significant value for t‡	Average blood sugar†	Difference	t calculated	Significant value for t‡
	Glycogen	Sugar									
hrs.				*per cent*	*per cent*			*mg. per 100 cc.*	*mg. per 100 cc.*		
Controls	12	12	N.	0.35 ±0.06				126 ±3.0			
"	15	11	D.	0.26 ±0.06	0.09	0.95	2.79	138 ±7.1	12	1.21	2.83
3	10	10	N.	2.31 ±0.10				162 ±8.0			
3	9	8	D.	1.80 ±0.08	0.51	3.86	2.90	176 ±6.0	14	1.15	2.92
6	19	19	N.	2.01 ±0.10				178 ±4.5			
6	10	10	D.	1.46 ±0.12	0.55	3.22	2.77	190 ±6.0	12	1.53	2.77
12	13	13	N.	1.32 ±0.09				169 ±8.0			
12	12	11	D.	1.49 ±0.09	0.17	1.21	2.81	145 ±6.0	24	2.10	2.82

* N. = normal; D. = deficient.

† Including the standard error of the mean.

‡ Based on a P value of 0.01 (chances of differences in result being due to errors in sampling 1 in 100).

thiamine-deficient animals should account for a certain amount of this effect. It is questionable whether it accounts for all of it. The blood sugar levels for these periods do not appear significantly different.

The results for the 12 hour period are particularly noteworthy. For, although the thiamine-deficient animals exhibited significantly

lowered hepatic glycogen at 3 and 6 hours after the glucose feedings, the comparison made 12 hours after this event reveals no significant difference between normal and deficient animals. In fact the latter group has maintained the liver glycogen at the same level as had been observed at the end of 6 hours, while the glycogen of the normal liver has decreased at the usual more or less regular rate in answer to the metabolic demands of the organism. Since the livers of normal animals had previously contained more glycogen than those from the deficient group, the observation that at 12 hours the values are quite similar is explainable on the basis of a difference in the rate of glycogenolysis. At this period, some degree of significance attaches to the differences in the blood sugar, since a comparison of the t values yields a P of less than 0.05.

These observations indicate that there is some retention of the hepatic glycogen in the thiamine-deficient animals, a phenomenon emphasized by Abderhalden and Wertheimer (20) as very characteristic of avitaminosis B in pigeons.

Glycogenesis after Lactate or Pyruvate—The observation previously mentioned that *in vitro* thiamine-deficient tissues exhibit a diminished ability to synthesize carbohydrate from pyruvate or remove lactate made it of interest to study this phenomenon *in vivo* as applied to hepatic glycogenesis.

Following a 48 hour fast, both normal and deficient test animals were fed 1 cc. of a 15 per cent solution of sodium $l(+)$-lactate per 100 gm. of body weight or 2 cc. per 100 gm. of body weight of a sodium pyruvate solution containing 100 mg. (calculated as pyruvic acid) of sodium pyruvate per cc. In these dosages no toxicity was observed and no marked intestinal disturbances were noted. A 6 hour period after the feeding of the salts was chosen for the removal of the liver, since Shapiro (21) had reported maximum glycogen formation at this interval.

Table III records the quantities of liver glycogen formed 6 hours after the feeding of these metabolites. A statistical appraisal of the apparent differences between the normal and thiamine-deficient animals indicates that there was no actual diminution of the ability of the avitaminotic organism to form liver glycogen from these intermediates. The lack of agreement between these results and the *in vitro* evidence (4) might be attributed to the fact that the degree of thiamine deficiency of these experiments was less pronounced than in the *in vitro* experiments of Barron and Lyman.

Ketolytic Effect of Glucose in Thiamine Deficiency—The ability of glucose to reduce the endogenous ketonuria following the development of a fatty liver in rats was a further aspect of the carbohydrate metabolism of thiamine-deficient animals to be studied. The experiments were carried out on female rats. Litter mates were selected and divided into two groups. One group was maintained on the normal stock diet; the other was reduced to a subacute state of thiamine deficiency. For a period of 2 weeks immediately preceding the experiments both groups were placed on a high fat diet similar to that used by Deuel, Hallman, and Murray (22): casein (vitamin B-free) 5 per cent, Crisco 38 per

TABLE III

Comparison of Liver Glycogen Formed 6 Hours after Feeding l(+)-Sodium Lactate or Sodium Pyruvate to Normal and Thiamine-Deficient Male Rats

Substance fed	Thiamine status	No. of experiments	Average glycogen*	Difference	t calculated	Significant value for t†
			per cent	*per cent*		
l(+)-Sodium lactate	Normal	11	1.48 ± 0.16			
	Deficient	10	1.18 ± 0.18	0.30	1.18	2.86
Sodium pyruvate	Normal	11	1.00 ± 0.10			
	Deficient	11	1.15 ± 0.18	0.15	0.68	2.84

* Including the standard error of the mean.

† Based on a P value of 0.01 (chances of difference in result being due to errors in sampling 1 in 100).

cent, glucose (cerelose) 43 per cent, Cellu flour 5 per cent, autoclaved yeast (Harris) 2 per cent, supplemented with riboflavin, pyridoxine, and variable amounts of thiamine as previously described.

For the production of a ketonuria the animals were fasted. During this time approximately half the members of each group were fed glucose in sodium chloride, by stomach tube, while the control group received only sodium chloride to induce diuresis. For the 1st day of the fast only salt was given. A ketolytic effect was induced by the administration twice daily of 0.5 cc. per 100 sq. cm. of body surface of a solution of glucose in 10 per cent sodium chloride containing 50 mg. of glucose per cc. Similar amounts of a 10 per cent sodium chloride solution were fed to the controls.

The urine was collected and diluted to 20 cc., representing a 24 hour sample. Total acetone bodies were determined by the Van Slyke procedure and urinary nitrogen by the Kjeldahl method. At the expiration of the 4 day experimental period, the animals were sacrificed and the livers removed for assay of the water and lipid content by procedures previously described (22).

Table IV records the ketonuria and urinary nitrogen obtained in the various experimental groups during the 2nd and 3rd days

TABLE IV

Acetone Body Excretion and Urinary Nitrogen in Mg. per 100 Sq. Cm. per Day

Thiamine status		No. of experiments	Body weight		Surface area*	Acetonuria†	$\frac{\text{M.d.}}{\text{S.e.m.d.}}$‡	Urine nitrogen†
			Start	End				
			gm.	*gm.*	*sq. cm.*			
Normal	Fasting controls; salt only	16	151.0	131.1	254.5	25.3 ±3.6		26.6 ±1.2
	Glucose fed	19	152.2	130.6	255.5	17.2 ±2.3	1.9	25.5 ±1.2
Deficient	Fasting controls; salt only	12	145.5	121.0	248.4	25.6 ±3.6		27.2 ±1.4
	Glucose fed	20	128.2	108.9	230.5	12.0 ±1.8	3.4	24.3 ±1.1

* Based on weight at start of fast. Calculated by the formula of Lee, surface area (sq. cm.) = $K \times W^{0.60}$ (23); $K = 12.54$; W = weight in gm.

† Including the standard error of the mean; 2nd and 3rd experimental days.

‡ Ratio of mean difference to the standard error of the mean difference. When this value exceeds 3.00, the results are considered significant.

of the fast. Because of a pronounced fall in the ketonuria on the 4th day these experiments are not considered. The quantity of glucose administered did not serve to reduce significantly the ketonuria of the normal animals but was effective in the case of the thiamine-deficient animals, although the extent of the ketonuria produced was very similar in both cases.

The results of the assay of the water and lipid content of the livers of the experimental animals at the conclusion of the 4 day fasting period yielded values for all the groups which were prac-

tically identical (19.9 to 23.2 per cent lipid). This indicates that the various experimental manipulations did not serve to alter these phases of metabolism.

SUMMARY

1. Certain aspects of carbohydrate metabolism *in vivo* were studied in animals reduced to a subacute state of thiamine deficiency.

2. When compared to normal animals, there was observed a decrease in the rate of absorption of glucose from the intestine and the extent of hepatic glycogenesis as well as the rate of glycogenolysis.

3. This subacute state of thiamine deficiency did not alter the ability of the animal to convert orally administered *l*(+)-sodium lactate or sodium pyruvate into hepatic glycogen.

4. In the thiamine-deficient animals, glucose was superior as a ketolytic agent for the reduction of an endogenous ketonuria produced after high fat diets.

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THE DENATURATION OF PROTEINS AND ITS APPARENT REVERSAL*

I. HORSE SERUM ALBUMIN

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Denaturation of certain globular proteins is accompanied by an increase in the relative viscosity of their solutions (2–4) and a decrease in their diffusion constant (2). These effects are indicative of changes in molecular dimensions and can be used for studying the denaturation of a protein and its apparent reversal (5–13).

For protein denaturation to be strictly reversible all changes occurring during denaturation (14–16) must be reversible. The present paper is mainly concerned with dimensional changes as determined by comparative diffusion and viscosity measurements on solutions of native, denatured, and "reversibly" denatured[1] serum albumin. Comparative electrophoretic, chemical, and immunological measurements are under way and will be presented in future publications.

EXPERIMENTAL

Material

Crystalline serum albumin Fractions A and B were prepared by the sodium sulfate method of Kekwick (17). The protein was

* Presented at the Thirty-fifth annual meeting of the American Society of Biological Chemists at Chicago, April 15–19, 1941 (1).

[1] The term "reversibly" denatured is employed here mainly in order to conform with the terminology of the literature and not as an accurate description of the process. In the present usage, "reversibly" denatured protein denotes that fraction which, following reversal of the conditions used for denaturation, approximates most closely the properties of the native protein.

preserved by desiccation from the frozen state (18) and dissolved when needed. Since it was soon found that the results obtained with Fraction A (containing 1.95 per cent carbohydrate) could be duplicated with Fraction B (0.08 per cent carbohydrate), most of the work described below was carried out with the former, as it was available in larger quantities. Evidently, the carbohydrate content has no relation to the processes studied here.

Urea (Merck) was recrystallized twice by dissolving in 70 per cent ethanol at 40° and chilling the saturated solutions to −9° (19). Aqueous solutions of the purified material were free from ammonia and had a pH of 6.8 to 7.0.

Guanidine hydrochloride (Eastman) was recrystallized twice at room temperature by adding 1 part of dry ether to 1 part of a saturated solution of the salt in absolute methanol.[2] The crystals were washed and dried for several days *in vacuo*. The concentrated solutions had a pH of 6.8.

Methods

Diffusion measurements were performed with the refractometric scale method at 25° ± 0.003°, as was described in previous publications from this laboratory (20, 21).

Density was measured in 5 cc. capped pycnometers at 25°.

Viscosity measurements were carried out at 25° ± 0.01° with the modified Ostwald viscometers described previously (22). For measurements under varying velocity gradients, pressure viscometers of the type described by Bingham and Jackson (23) were used. They were calibrated with water and the measurements corrected for the contribution of the kinetic energy. The average velocity gradient could be varied between 175 and 2000 sec.$^{-1}$. A pressure control apparatus of the type described by Bingham (24) allowed the external pressures to be kept constant to within 1 mm. of water during the measurements.

Results

Denatured Serum Albumin in Urea and Guanidine Hydrochloride—The diffusion constants and viscosities of serum albumin have been found previously (2) to change gradually with increasing concentration of urea up to about 6.7 M. In the present work,

[2] Personal communication of Dr. J. P. Greenstein.

the denaturing power of urea and that of guanidine hydrochloride were compared with one another in 8 M solutions,[2] in the presence of acetate buffer of pH 5.0 containing 0.023 M acetate and 0.2 M NaCl.

The relative viscosities were determined in protein concentrations between 0.1 and 1.1 per cent and are plotted in Fig. 1 against

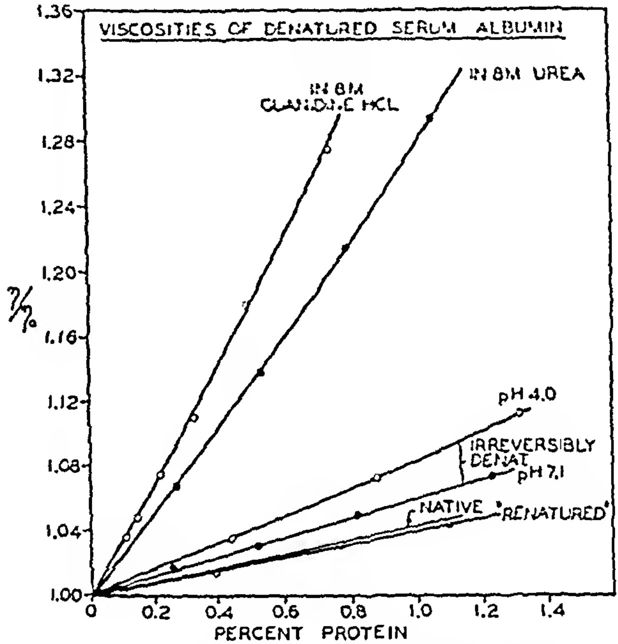

FIG. 1. Relative viscosities of serum albumin plotted against the protein concentration in weight per cent. The curves refer to, in order of decreasing slopes, the denatured serum albumin in 8 M guanidine hydrochloride and in 8 M urea, the irreversibly denatured protein at pH 4.0 and pH 7.1, the native serum albumin, and the "reversibly" denatured ("renatured") serum albumin.

concentrations expressed in weight per cent. The limiting slopes of the curves were determined from the intercept when η_{sp}/c

[2] The concentrations are expressed here in molarities. The values given by Greenstein approximate molalities rather than molarities inasmuch as the solutions were prepared by adding a given amount of the substance to a given volume of the solvent (14).

was plotted against c, where η_{sp} is the specific viscosity and c the concentration (25). Comparative measurements in the pressure

TABLE I

Diffusion Constants of Denatured, "Reversibly" Denatured, and Irreversibly Denatured Serum Albumin

t = time in seconds; D = average diffusion constant in sq. cm. per second; D' = diffusion constant corrected for the viscosity of the solvent with Equation 1; D_1, D_2, and D_3 are the diffusion constants calculated by the maximum height, standard deviation, and successive analysis methods respectively. Unless otherwise indicated, each D_3 value is the mean of about six values determined from evenly spaced parts of the diffusion curves (22).

Concentration	t	D_1	D_2	D_3
Serum albumin in 8 M urea				
per cent	*sec.*	10^{-7}	10^{-7}	10^{-7}
1.2	52,320	2.36		2.32
	76,680	2.26		2.38
0.8	32,640	2.15		
	75,660	2.21	2.23	
Average.....		$2.27 \times 10^{-7} \pm 0.09$		
D'..........		4.13×10^{-7}		
Serum albumin in 8 M guanidine hydrochloride				
1.1	64,080	1.47		1.44
0.8	21,540	1.33		1.31
	44,820	1.46		
	79,440	1.44		
Average		$1.46 \times 10^{-7} \pm 0.08$		
D'..........		3.74×10^{-7}		

Concentration	t	D_1	D_2	D_3
Serum albumin "reversibly" denatured by 8 M urea				
per cent	*sec.*	10^{-7}	10^{-7}	10^{-7}
1.2	32,760	7.08		6.98
	66,420	6.68		7.11
0.8	20,520	7.09		7.15
	40,560	7.11		
	105,600	6.75		
Average........		$6.98 \times 10^{-7} \pm 0.22$		
D'............		7.17×10^{-7}		
Irreversibly denatured by 8 M urea, at pH 7.1				
0.8	32,820	4.27		
	52,980	4.21	4.11–5.60	
	81,600	4.00	5.18	
Limiting value.		5.60×10^{-7}		
D'............		5.74×10^{-7}		
Irreversibly denatured by 8 M urea, at pH 4.0				
0.4	24,960	3.83	Indefinite	
	35,340	3.71		
0.2	21,240	3.16		

viscometer indicated the relative viscosities to be independent of the velocity gradient between 175 and 2000 sec.$^{-1}$, in agreement with similar findings by Greenstein (26).

The results of the diffusion measurements are given in Table I. Apparent molecular shapes of the denatured protein were calculated from the limiting slopes of the viscosity curves, with the Simha equation (27), assuming the molecular weight of the denatured protein to be the same in urea and guanidine as that of the native protein in aqueous solution (28) (Table II). The apparent molecular shape was also calculated from the observed diffusion constant, corrected for the viscosity of the solvent, and the spherical diffusion constant D_0, with Perrin's equation (22). The results indicated that the asymmetries calculated from diffusion were consistently higher than those derived from viscosity data. If both sets of data are expressed in terms of dissymmetry constants, f/f_0 (29), the constants evaluated from the diffusion data, $(f/f_0)_D$, exceed those evaluated from viscosities, $(f/f_0)_\eta$, by a factor of 1.1. Hence, the molecular weights calculated from diffusion and viscosity measurements in concentrated urea or guanidine hydrochloride solutions, when the method discussed in detail previously (22) is applied, are about 50 per cent higher than those found by osmotic pressure measurements. The origin of this discrepancy, already noted by Neurath and Saum (2), has been traced in the present work to the viscosity correction of the diffusion constants when measured in solutions of high viscosity.

$$D' = D \frac{\eta}{\eta_0} \tag{1}$$

where D is the measured diffusion constant, η/η_0 the measured relative viscosity of the solvent, and D' the corrected diffusion constant. A solution of native serum albumin in 10 per cent sucrose containing the same acetate buffer used for the measurements in urea or guanidine hydrochloride was prepared and allowed to diffuse against a solvent of the same composition. Diffusion was measured with 0.9 and 0.7 per cent protein solutions. The observed diffusion constant was found to be $4.86 \times 10^{-7} \pm 0.13$ (standard deviation of the mean from six values), which, corrected for the relative viscosity of the solvent ($\eta/\eta_0 = 1.327$), yields a value of 6.43×10^{-7}, as compared with a value of 6.99×10^{-7} in the absence of sucrose. Yet the limiting value for η_{sp}/c was found to be 4.30 in 10 per cent sucrose as well as in buffer solution. These results suggest that the normal viscosity correction (Equation 1) is not

applicable when the viscosity of the solvent is high, and furnish an experimental explanation for the discrepancies noted above.[4] When this empirical correction factor was applied to the data,

TABLE II

Molecular Constants of Native, Denatured, "Reversibly" and Irreversibly Denatured Serum Albumin Fractions

η_{sp}/c = the limiting slope of the curves obtained when the specific viscosity is plotted against the protein concentration; b/a = the ratio of the axes for a prolate ellipsoid calculated with the Simha viscosity equation and neglecting solvation; $(b/a)_h$ = the ratio of the axes, assuming 33 per cent hydration; D' = the diffusion constant corrected for the viscosity of the solvent with Equation 1; $(f/f_0)_\eta$ = the dissymmetry constant calculated from viscosity data; $(f/f_0)_D$ = the dissymmetry constant from diffusion data, assuming a molecular weight of 70,000; and M = the molecular weight calculated from diffusion and viscosity data.

Protein	$\frac{\eta_{sp}}{c}$	$\frac{b}{a}$	$\left(\frac{b}{a}\right)_h$	D'	$\left(\frac{f}{f_0}\right)_\eta$	$\left(\frac{f}{f_0}\right)_D$	M
				10^{-7}			
Native	4.30	5.0	3.3	6.99	1.25	1.27	71,900
Denatured in 8 M urea	22.25	16.8	13.3	4.13	1.86	2.12	77,800*
Denatured in 8 M guanidine hydrochloride	31.55	20.9	16.7	3.74	2.03	2.34	80,600*
"Reversibly" denatured by 8 M urea	4.10	4.7	3.1	7.17	1.23	1.22	70,300
Irreversibly denatured by 8 M urea, at pH 7.1	5.90 (Average)	6.6 (Average)	4.7	5.74 (Limiting value)			104,000 (Average)

* Molecular weight calculated with the empirical correction for the diffusion constant (see the text).

values for the molecular weight of the denatured protein in urea and guanidine hydrochloride were obtained as listed in Table II. In Table II there are also listed values for the molecular shape, $(b/a)_h$, calculated for 33 per cent hydration.

[4] Further studies on this effect are in progress.

Distribution between "Reversibly" and Irreversibly Denatured Serum Albumin—When solutions of serum albumin in concentrated urea are dialyzed in the cold against distilled water and subsequently exposed to room temperature, a precipitate appears, firmly adhering to the walls of the container. Further investigation led to the following method for optimal separation of soluble and insoluble protein. A 2 per cent protein solution in a given concentration of urea or guanidine hydrochloride was allowed to stand at room temperature for about 20 hours. The solutions were then dialyzed against running water at 4–5° until the dialysate was free from urea, or guanidine hydrochloride. They were then adjusted to pH 5.25 (with 0.2 N sulfuric acid) and exposed for 30 minutes to 41° in a water bath, resulting in precipitation of the insoluble fraction. Longer heating of the supernatant solution at 41° did not produce further precipitation. The precipitate was collected by centrifugation, washed once with distilled water at 41°, and then dissolved at pH 4 or 7.1.

Separation could also be accomplished by fractional precipitation with sodium sulfate. It was found that, after the denaturing agent was dialyzed out, a fraction was precipitated from a 3 per cent solution at pH 4.9 when the sodium sulfate concentration was made 20 gm. per 100 cc. of solution, whereas 23.5 gm. of salt were needed to precipitate the remaining protein from the supernatant solution. The quantitative distribution obtained by the salt method was the same as that obtained by the heating method. In view of the solubility properties of these two fractions, as compared with those of the native protein, we shall, in the following, refer to the insoluble fraction as irreversibly denatured and to the soluble fraction as "reversibly" denatured protein.

The distribution between the two fractions, as a function of the concentration of the denaturing agent originally present, was investigated by preparing 10 cc. samples of 2 per cent protein in 2, 4, 6, and 8 M urea, or guanidine hydrochloride, and using the method of heat separation for analysis. Protein concentration was determined by the Koch-McMeekin method (30). The results of triplicate determinations are given in Fig. 2 in which per cent of total protein irreversibly denatured is plotted against the concentration of denaturing agent originally present. It will be noted that the maximum yield of 15 per cent irreversibly denatured

protein is independent of the urea concentration above 6 M. The same limiting value is reached in guanidine hydrochloride concentrations above 2 M.

"Reversibly" Denatured Serum Albumin—In order to investigate the properties of the "reversibly" denatured material, a large batch of serum albumin in 2 per cent solution was denatured by 8 M urea and the irreversibly denatured fraction removed by the method of heat treatment at 41°. Diffusion measurements on the unfractionated supernatant protein solution in the presence of 0.023 M acetate buffer at pH 5.0, containing 0.2 M NaCl, revealed the presence of more than one component in these solutions. The mean diffusion constant, calculated by the maximum height method ((22) Equation 6) was found to be 6.0×10^{-7}, as compared with a value of 7.0×10^{-7} for native material, whereas calculations by the method of successive analysis ((22) Equation 8) yielded a limiting value of 7.0×10^{-7} in the region below their inflection points. Viscosity measurements on this material yielded a limiting value of η_{sp}/c of 4.50 as compared with 4.30 for the native protein.

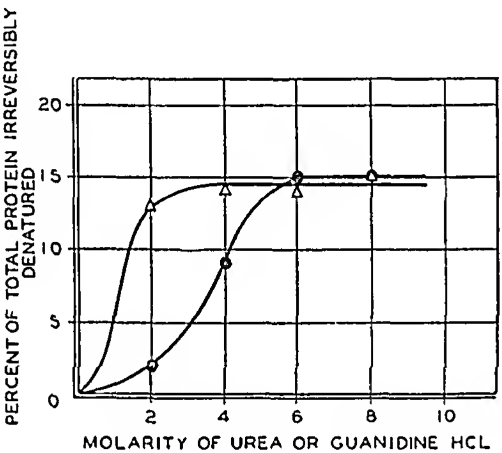

FIG. 2. The fraction of total serum albumin irreversibly denatured after dialysis, plotted against the molarity of urea or guanidine hydrochloride at which denaturation occurred. *The circles refer to urea; the triangles to guanidine hydrochloride.*

In order to purify the "reversibly" denatured serum albumin, the protein was subjected to fractional precipitation with sodium sulfate. After it was ascertained that heating of solutions of native

material to 41° did not impair its ability to crystallize, although it changed the crystal form to that of long, narrow needles, 100 cc. of a 3 per cent solution of "reversed" denatured serum albumin were adjusted to pH 4.9 and solid sodium sulfate added slowly with stirring. Increasing the salt concentration up to 20 gm. per 100 cc. resulted in the precipitation of small amounts of amorphous material. Upon further addition of 3.5 gm. of sulfate (a total of 23.5 gm.) to the filtrate a bulky precipitate of *amorphous* protein settled out. The final filtrate contained only traces of protein. Analogous results were obtained with serum albumin Fraction B, or when precipitation was carried out at pH 6.0 instead of at 4.9. Similarly, the precipitate was amorphous if the irreversibly denatured protein was first removed by salt precipitation instead of by heating to 41°.[5]

The fraction precipitating at a concentration of 23.5 gm. of Na_2SO_4 per 100 cc. of solution was dissolved in water, dialyzed, and subjected to diffusion and viscosity measurements in the presence of acetate buffer of pH 5.0. The results of the diffusion measurements listed in Table I indicate that the material is essentially monodisperse. The diffusion constant of 7.17×10^{-7} is somewhat higher than that of the native protein, whereas the limiting slope of the viscosity curves reproduced in Table II

[5] Anson and Mirsky (9) reported the crystallization of serum albumin after reversing the denaturation by acid acetone. Since little is known about the comparative effects of acid acetone and urea, or guanidine hydrochloride, no conclusions can be drawn from the discrepancy between Anson and Mirsky's findings and those reported here. However, it has been found possible to obtain a minor part of the "reversibly" denatured material in crystalline form. If the amorphous precipitate is redissolved in water and dialyzed, an amorphous fraction precipitates after 22.5 gm. of solid sodium sulfate have been added per 100 cc. of a 3 per cent protein solution at pH 4.9. After the salt concentration of the supernatant is raised to 25 gm. per 100 cc., a crystalline precipitate is obtained, the crystals being different from those of the native protein heated to 41°. They have the shape of small, pointed needles, clustering together in a fan-shaped manner, and resembling closely those of Bence-Jones protein. The protein remaining in the supernatant solution is amorphous when precipitated by increasing the salt concentration by slow evaporation. Our findings differ from those of Burk (28) who obtained crystals from serum albumin reversibly denatured by 6.66 M urea, indistinguishable from those of the native protein.

(η_{sp}/c = 4.10) is somewhat lower than the value found for the native protein. The molecular weight of 70,300 (Table II) calculated from diffusion and viscosity data agrees with that of native serum albumin.

Irreversibly Denatured Serum Albumin—The precipitate of irreversibly denatured protein, obtained by either heat treatment or fractional precipitation with salt, was of jelly-like consistency and, when suspended in dilute acid at pH 4, in the absence of salts, swelled up like gelatin before going into solution. After any undissolved material was centrifuged off, the viscous solution was adjusted back to pH 5.25; the precipitated protein was washed with distilled water and redissolved at the desired pH. Viscosity measurements were carried out in 0.025 N phosphate buffer at pH 7.1 containing 0.1 N NaCl, in 0.05 N veronal-acetate buffer at pH 7.5 containing 0.1 N NaCl, and in 0.05 N acetate buffer at pH 4.0 containing 0.1 N NaCl. The results of the viscosity measurements are plotted in Fig. 1. The points obtained at pH 7.5 fell on the line shown here for pH 7.1, whereas the slope of the curve obtained at pH 4.0 was higher. The relative viscosity at pH 7.5 was independent of the velocity gradient as measured in the pressure viscometer.

The results of the diffusion measurements are summarized in Table I. From the limiting value of the diffusion constant, measured at pH 7.5 and calculated by the method of successive analysis, and the mean value of η_{sp}/c, a mean molecular weight of about 104,000 was calculated (Table II), indicating the occurrence of irreversible aggregation. The lower diffusion rate at pH 4.0 was probably caused by a retardation in the diffusion rate at this pH due to gel structure.[6]

DISCUSSION

The present viscosity and diffusion measurements of denatured serum albumin in the presence of urea or guanidine hydrochloride agree with the findings previously reported by Neurath and Saum

[6] The solutions do not exhibit double refraction of flow in the simple apparatus described by Edsall and Mehl (31) nor does the protein sediment in the analytical centrifuge under the influence of a centrifugal field 47,000 *g*. Measurements of the viscosity in a modified Couette viscometer will be reported later.

(2). The discrepancies between molecular weights calculated on the one hand from diffusion and viscosity data and on the other from osmotic pressure determinations (28) are greatly reduced if the viscosity data are interpreted with the Simha equation and if for diffusion measurements, in solvents of relatively high viscosity, the empirical correction factor is introduced.

The changes in viscosity and diffusion accompanying denaturation may be ascribed to changes in molecular volume, shape, or both (2). If the effects observed for the urea denaturation were to be interpreted solely in terms of a combination between serum albumin and urea, solvation to the extent of about 2.7 gm. of urea per gm. of protein would have to be assumed, equivalent to about 3200 molecules of urea per protein molecule. If, however, the changes in relative viscosity and diffusion constant are ascribed entirely to an increase in molecular asymmetry, values are obtained as given in the fourth column of Table II. The interpretation in terms of increased asymmetry receives support from the observed increase in the relative viscosity of egg albumin (3) and hemoglobin (4) following denaturation by heat, a reaction which, if anything, causes a decrease in the hydration of the protein (32). Furthermore, the formation of fibrous structures has been observed in urea denaturation of edestin and other proteins (33), and surface denaturation of a number of globular proteins is known to increase the asymmetry to the extent that the molecules unfold into structures closely resembling open polypeptide chains (34).

The present measurements indicate a stronger denaturing power of guanidine hydrochloride as compared with equimolar concentrations of urea, a conclusion already reached by Greenstein from measurements on protein sulfhydryl groups (14).

The extent of apparent reversal of denaturation, as determined by differences in solubility of "reversibly" and irreversibly denatured material, is a function of the concentration of urea or guanidine hydrochloride at which denaturation has occurred. An explanation for this phenomenon may be sought in any one of the following hypotheses. (1) Denaturation has been incomplete and the fraction which appears to be "reversibly" denatured has not been denatured in the first place. (2) Serum albumin consists of two components of closely related properties, one of them being capable of "reversible" denaturation, the other one not. (3) The

solutions of denatured protein are homogeneous and separation into two fractions occurs as a result of removal of the denaturing agent by dialysis. If the first hypothesis were correct, 85 per cent of the total protein present in 8 M guanidine hydrochloride solution should be in the native state and should exhibit a diffusion constant 70 per cent higher than that observed. As the presence of two components of such widely different diffusion constants can be readily detected experimentally, this hypothesis appears to be discredited by the observed monodispersity of the solutions. If the second hypothesis were correct, redenaturation of the "reversibly" denatured fraction should be totally reversible. Instead it was found that the "reversibly" denatured protein had an equal, if not higher susceptibility to irreversible denaturation than the native, 20 and 19 per cent being irreversibly denatured by 4 and 6 M guanidine hydrochloride respectively, as compared with about 14 per cent for the analogous experiments with the native material. These facts, incongruous with the first two hypotheses, appear to lend support to the third.

The sigmoidal shape of the curve obtained when the fraction of total protein irreversibly denatured is plotted against the concentration of the denaturing agent (Fig. 2) suggests a statistical interpretation. Assuming that in the present case each concentration of urea or guanidine hydrochloride represents a certain stage in the unfolding[7] process of the protein molecules, as evidenced by the increase in molecular asymmetry, one may conclude that the greater the extent of unfolding the lower will be the probability that all molecules will find their way back to the condensed configuration. If the distortion in molecular configuration is small, as for instance, in 2 M urea solutions, "reversal" of denaturation will be practically complete (2 per cent irreversibly denatured). As the unfolding process progresses, a higher fraction of the total protein will escape "reversal" until, in concentrations of urea higher than about 6 M, the extent of unfolding will approach a limiting maximum level and the probability of "reversal" its minimum (equivalent to 85 per cent of total protein).

[7] Whether the denatured molecule actually consists of an aggregation of disoriented polypeptide chains (33) or of an extended structure intermediate between that of polypeptide chains and the condensed configuration of the native molecule cannot be decided without further experimental evidence.

Previous evidence adduced in favor of "reversible" denaturation of serum albumin by heat or acid acetone (9, 10, 35) has been questioned by Hewitt (36) for insufficient proof of the protein having been denatured under the conditions employed. This criticism can hardly be applied to the present investigation, since the extent of denaturation was followed by measurements on the denatured protein in the presence of the denaturing agent.

While diffusion and viscosity measurements failed to indicate any significant differences between native serum albumin and the material obtained after reprecipitation of the "reversibly" denatured fraction, nevertheless, other observations cast some doubt on the strict reversibility of the denaturation of this protein. It was noted that the tendency and conditions for crystallization changed upon "reversal" of the denaturation and that the allegedly reversibly denatured protein had a somewhat higher solubility in sodium sulfate than the native. Also, electrophoretic measurements in the Tiselius apparatus, to be reported in detail elsewhere,[8] showed differences in electrophoretic mobility.

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SUMMARY

Denaturation of crystalline horse serum albumin by 8 M solutions of urea, or guanidine hydrochloride, results in a large increase in relative viscosity and a decrease in diffusion constant. In equimolar solutions, guanidine hydrochloride is a more effective denaturing agent than urea. The diffusion constant decreases in proportion to the increase in relative viscosity if a retardation of the diffusion rate by a constant factor is taken into account. The molecular weight of serum albumin does not appear to change during denaturation.

Upon removal of the denaturing agent by dialysis, the protein separates into two fractions differing from one another in solubility. The more soluble fraction has been recognized as apparently "reversibly" denatured protein, whereas the more in-

[8] Sharp, D. G., Cooper, G. R., Erickson, J. O., and Neurath, H., unpublished data.

soluble fraction is composed of irreversibly denatured material. The fraction of total protein irreversibly denatured increases in a sigmoidal manner with the urea or guanidine hydrochloride concentration at which denaturation occurred, reaching a maximum value of 15 per cent in 6 M urea, or 2 M guanidine hydrochloride. This behavior is believed to reflect a probability factor governing the reformation of globular protein molecules from the denatured state.

The protein obtained after fractional precipitation of the more soluble fraction with salt has about the same molecular weight and about the same apparent molecular shape as the native material. It differs from the latter, however, in solubility in sodium sulfate, in crystallizability, and in electrophoretic mobility. This suggests reversal of denaturation to be only apparent and not quantitative.

The protein of the irreversibly denatured fraction is insoluble at the isoelectric point and is polydisperse when dissolved in solutions of pH 7.1. In slightly acid solutions it exhibits an apparent tendency for gel formation.

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THE DENATURATION OF PROTEINS AND ITS APPARENT REVERSAL*

II. HORSE SERUM PSEUDOGLOBULIN

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In the preceding paper (2) the denaturation of horse serum albumin by urea and guanidine hydrochloride and its apparent reversal were described. These studies have been extended in the present investigation to pseudoglobulin components with the object of determining any differences that may exist in the denaturation process of the protein constituents of normal horse serum. Quantitative studies have been made possible by recent improvements in the experimental methods of isolating monodisperse serum pseudoglobulin fractions (3, 4). Their known physical and chemical characteristics serve as a sensitive criterion for the extent to which denaturation can be reversed.

EXPERIMENTAL

Material

The method of preparation of monodisperse fractions of serum pseudoglobulins by means of fractional precipitation with ammonium sulfate under defined experimental conditions relative to protein concentration, pH, and salt concentration has been described in a previous publication (3). The present measurements were carried out by the technique already described (2) with the pseudoglobulins GI and GII, precipitable by ammonium sulfate at pH 6.4 within the limits of 1.1 to 1.36 and 1.4 to 1.6 M respectively. The molecular weights of GI and GII were found

* Presented at the Thirty-fifth annual meeting of the American Society of Biological Chemists at Chicago, April 15-19, 1941 (1).

by diffusion and viscosity measurements to be 170,000, in satisfactory agreement with the values of 165,000 obtained by Tiselius for electrophoretically isolated material (5), and of 178,000 found by Burk (6) from osmotic pressure measurements.[1]

Since it was found early in this work that no fundamental differences existed between the fractions GI and GII relative to the denaturation process as studied here, most of the measurements described below were carried out with the pseudoglobulin GII, as it was available in larger quantities.

Results

Denatured Pseudoglobulin in Presence of Urea and Guanidine Hydrochloride—The diffusion and viscosity of pseudoglobulin were measured in the presence of different amounts of urea and guanidine hydrochloride in solutions containing 0.05 N acetate buffer and 0.2 M NaCl at pH 5.5. The results of the viscosity determinations carried out at protein concentrations between 0.1 and 1.2 per cent are plotted in Fig. 1.

As in the analogous studies on serum albumin (2), the relative viscosities increase with increasing concentration of the denaturing agent, the increase being greater for guanidine hydrochloride than for comparable concentrations of urea. The limiting slopes of the curves were determined from the intercept when η_{sp}/c was plotted against c, where η_{sp} is the specific viscosity and c the protein concentration in weight per cent (7). Comparative measurements in the pressure viscometer showed the relative viscosities to be independent of the velocity gradient within the region of 175 to 2000 sec.$^{-1}$.

Diffusion constants were measured in conjunction with the viscosity determinations. The results are summarized in Table I.

Analysis of the diffusion curves indicated the solutions to be essentially monodisperse in urea concentrations of 5 and 8 M, and in guanidine hydrochloride concentrations of 2 and 5.6 M. In 0.5 and 3 M guanidine hydrochloride there is some spreading of the values as determined by the method of successive analysis, indicating the presence of a small fraction of lower diffusion constant.

Calculations of apparent molecular shapes and molecular weights

[1] Lower values, *i.e.* 142,000, have been reported by Cohn *et al.* (4) for material isolated by methods similar to those employed by the authors (3).

from diffusion and viscosity data were carried out as described in the preceding paper (2) and are summarized in Table II. There are also included values for the molecular shape, $(b/a)_A$, calculated with the assumption of 33 per cent hydration. The empirical

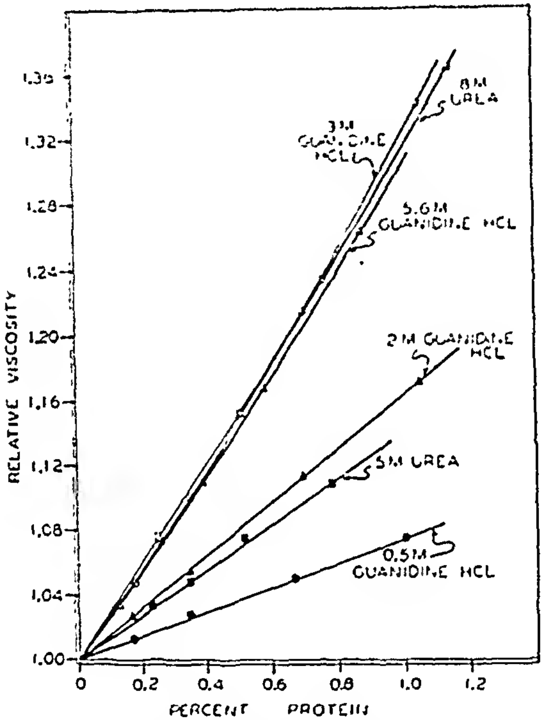

Fig. 1. Relative viscosities of pseudoglobulin denatured by urea and guanidine hydrochloride, plotted against protein concentration in weight per cent. Open circles refer to 3 M guanidine hydrochloride, open squares to 8 M urea, open triangles to 5.6 M guanidine hydrochloride, solid circles to 0.5 M guanidine hydrochloride, solid squares to 5 M urea, and solid triangles to 2 M guanidine hydrochloride.

viscosity correction for the diffusion constant (2) was applied to the measurements in urea solutions and to the measurements in guanidine hydrochloride in concentrations higher than 2 M. In cases in which the solutions were found to be polydisperse, the limiting value of the diffusion constant was used for molecular weight calculations.

TABLE 1

Diffusion Constants of Denatured and "Reversibly" and Irreversibly Denatured Pseudoglobulin*

t = time in seconds; D = mean diffusion constant in sq. cm. per second; D' = diffusion constant corrected for the viscosity of the solvent ((2) Equation 1); D_1, D_2, and D_3 are the diffusion constants calculated by the maximum height, standard deviation, and successive analysis methods, respectively. Unless otherwise indicated, each D_3 value is the mean of about six values determined from evenly spaced parts of the diffusion curves (3).

Concentration	t	D_1	D_2	D_3
Pseudoglobulin in 0.5 M guanidine HCl				
per cent	*sec*	*10^{-7}*	*10^{-7}*	*10^{-7}*
0.5	23,640	4.16		4.02
	34,980	4.02		
	82,140	3.91		3.93–4.42
Limiting value		4.4×10^{-7}		
D'		4.6×10^{-7}		
Pseudoglobulin in 2 M guanidine HCl				
0.55	24,720	2.76		
	32,640	2.86		
	41,580	2.87		
	73,380	2.79		2.89
Average		$2.85 \times 10^{-7} \pm 0.05$		
D'		3.19×10^{-7}		
Pseudoglobulin in 3 M guanidine HCl				
0.8	20,520	2.88		
	20,400	2.67		
	27,000	2.72		
	64,200	2.63		2.58–3.04
	81,120	2.75		2.61–3.07
Limiting value		3.0×10^{-7}		
D'		3.7×10^{-7}		
Pseudoglobulin in 5.6 M guanidine HCl				
1.2	12,780	2.50		2.47
	86,910	2.51		
0.8	37,080	2.45		
Average		$2.50 \times 10^{-7} \pm 0.11$		
D'		4.02×10^{-7}		
Pseudoglobulin in 5 M urea				
per cent	*sec.*	*10^{-7}*	*10^{-7}*	*10^{-7}*
1.2	38,640	2.97		
	75,000	2.75		2.72
	104,400	2.77		
	164,100	2.65		
Average		$2.76 \times 10^{-7} \pm 0.12$		
D'		3.73×10^{-7}		
Pseudoglobulin in 8 M urea				
1.2	86,400	1.76		
	109,440	1.68	1.79	
	132,420	1.75		
	51,165	1.65		
	79,380	1.73		1.89
	103,680	1.62		
Average		$1.70 \times 10^{-7} \pm 0.09$		
D'		2.97×10^{-7}		
Pseudoglobulin, "reversibly" denatured by 8 M urea				
0.7	35,760	4.56		4.50
	45,600	4.44		
	59,940	4.49		
	71,940	4.61		4.55
Average		$4.53 \times 10^{-7} \pm 0.12$		
D'		4.69×10^{-7}		
Pseudoglobulin, irreversibly denatured by 7.5 M urea, at pH 7.5				
0.33	40,740	0.79		
	60,300	0.87		
	133,680	0.84		0.91–1.50

* See (2), foot-note 2.

Distribution between "Reversibly" and Irreversibly Denatured Pseudoglobulins—Removal of urea or guanidine hydrochloride by dialysis against distilled water in the cold resulted in partial precipitation of the proteins. The precipitate was of gelatinous consistency varying in amount with the pH of the suspension and with

TABLE II

Molecular Constants of Native, Denatured, and "Reversibly" Denatured Pseudoglobulins

η_{sp}/c = the limiting slope of the curves obtained when the specific viscosity is plotted against protein concentration; b/a = the ratio of the axes for a prolate ellipsoid, calculated with the Simha viscosity equation, solvation being neglected; $(b/a)_h$ = the axial ratio calculated for 33 per cent hydration; D' = the diffusion constant corrected for the viscosity of the solvent ((2) Equation 1): (f/f_0) = the dissymmetry constant calculated from viscosity data; and M = the molecular weight calculated from diffusion and viscosity data.

Protein	$\frac{\eta_{sp}}{c}$	$\frac{b}{a}$	$\left(\frac{b}{a}\right)_h$	D' 10^7	$\frac{f}{f_0}$	M
Native........	6.60	7.2	5.2	4.75	1.39	170,000
In 0.5 M guanidine HCl	7.75	8.1	6.0	4.6*	1.44	170,000*
" 2 M guanidine HCl	14.90	13.0	10.0	3.49	1.69	307,000
" 3 " " " .	27.0	19.0	15.0	3.7*	1.95	95,900*†
" 5.6 M guanidine HCl . .	27.0	19.0	15.0	4.02	1.95	74,800†
" 5 M urea........... .	14.0	12.4	9.3	3.73	1.66	152,000†
" 8 " "	28.0	19.5	15.1	2.97	1.98	170,000†
"Reversibly" denatured by 8 M urea.........	5.90	6.6	4.7	4.69	1.35	190,000

* The solution was somewhat polydisperse (see Table I). The values refer to the limiting diffusion constant determined by the method of successive analysis.

† Molecular weight calculated with the empirical correction for the diffusion constant (see the text).

temperature. Maximum yield was obtained when solutions were adjusted to pH 6.0 and stored at about 0°.

The quantitative distribution between insoluble (irreversibly denatured) and soluble ("reversibly" denatured) pseudoglobulin was studied as a function of the concentration of the denaturing agent originally present. 10 cc. samples of 2 per cent protein in 2, 4, 6, 7, and 8 M urea or guanidine hydrochloride were prepared

and, after 12 hours, dialyzed in the ice box against running distilled water until free from salt. The solutions were next adjusted to pH 6.0 and stored overnight at 0°. The precipitates were then centrifuged, washed once with distilled water, dissolved in a phosphate buffer of pH 7.1, and made to volume. The supernatants and washings were likewise made to volume and the pro-

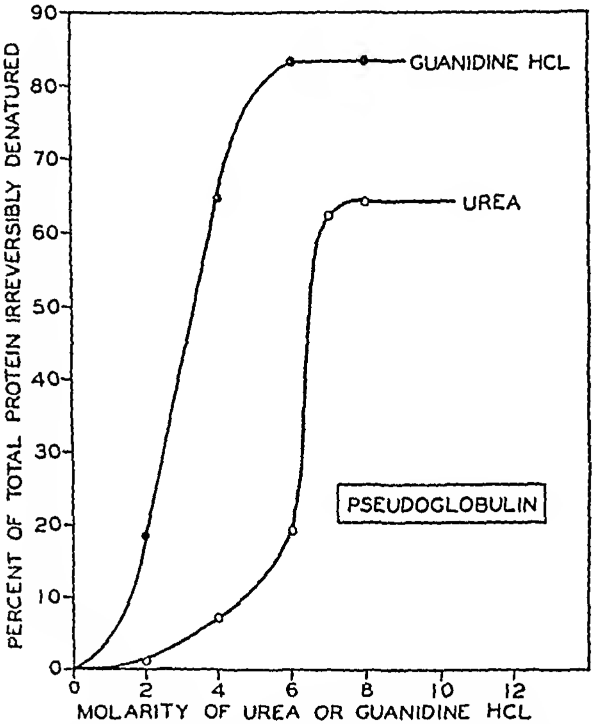

FIG. 2. The fraction of total pseudoglobulin irreversibly denatured after dialysis, plotted against the molarity of urea or guanidine hydrochloride at which denaturation occurred. Open circles refer to urea, solid circles to guanidine hydrochloride.

tein concentrations in the respective fractions determined with the Koch-McMeekin method (8). The results of these measurements, carried out in triplicate, are given in Fig. 2 in which the fraction of total protein irreversibly denatured is plotted against the concentration of urea, or guanidine hydrochloride originally present.

"Reversibly" Denatured Pseudoglobulin—In the following experiments, a 2 per cent protein solution was denatured by 8 M urea

and, after standing for 24 hours, urea was removed by dialysis. Diffusion measurements on the supernatant solution, obtained after precipitation of the irreversibly denatured protein at pH 6.0, indicated the material to be polydisperse. The diffusion constants, measured in the presence of an acetate buffer of pH 5.5, varied between 3.8×10^{-7} near the peak of the diffusion curves and 4.6×10^{-7} in the lower regions. The limiting slope of the viscosity curves, η_{sp}/c, was found to be higher than that of the native globulin; *i.e.*, 7.90 as compared with 6.60.

For further purification, the proteins contained in the supernatant solution were subjected to fractional precipitation with ammonium sulfate, according to the method employed for the purification of the native protein: the protein concentration was adjusted to 3 per cent, the pH to 6.4, and the ammonium sulfate concentration gradually increased to 1.1 M. At this point the solution became slightly opalescent and when more ammonium sulfate was added, up to 1.36 M, a precipitate settled out. After filtration, the salt concentration was raised to 1.6 M; the precipitate collected by centrifugation, dialyzed, and, after removal of traces of euglobulin by pH adjustment to 6.2 and 5.0, used for diffusion and viscosity measurements in the presence of a 0.05 M acetate buffer, pH 5.5, containing 0.2 M NaCl.

The results of the diffusion measurements are listed in Table I. The material proved to be monodisperse with a diffusion constant of $D = 4.69 \times 10^{-7}$ with a standard deviation of the mean of $\pm 0.12 \times 10^{-7}$. The limiting slope of the viscosity curves, illustrated in Fig. 3, was 5.90 as compared with 6.60 for native material. The molecular weight of 190,000 calculated from these data is somewhat higher than that found for the native material (Table I).

Irreversibly Denatured Pseudoglobulins—The fraction which precipitated upon adjustment of the pH to 6.0, following removal of 8 M urea by dialysis, was considered to be irreversibly denatured material. For further purification, it was dissolved by acidifying to pH 4.0 and freed from any insoluble residue by filtration. In a concentration of 2 per cent protein, the solution was highly viscous and appeared to exhibit thixotropic properties. It did not show double refraction of flow in the apparatus described by Edsall and Mehl (9). The protein was reprecipitated by adjustment of the

pH to 6.0 and then redissolved at the desired pH. Addition of the buffer components by dialysis resulted in partial precipitation. The results of the viscosity measurements carried out in a 0.05 N acetate buffer at pH 4.12, containing 0.1 N NaCl, and in a 0.02 N veronal-acetate buffer at pH 7.5, containing 0.1 N NaCl, are shown in Fig. 3. The relative viscosities were also measured in the pressure viscometers and found to be independent of the veloc-

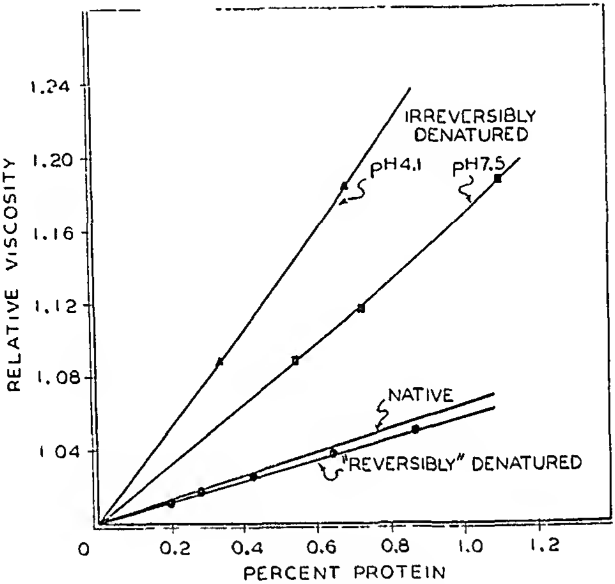

FIG. 3. Relative viscosities of irreversibly and "reversibly" denatured pseudoglobulin, plotted against the protein concentration in weight per cent. Triangles refer to irreversibly denatured protein at pH 4.1, squares to the same protein at pH 7.5, circles to "reversibly" denatured protein, purified by fractional precipitation with ammonium sulfate (see the text). For comparison, the slope of the viscosity curve of the native protein is also indicated.

ity gradient[2] between 400 and 2500 sec.$^{-1}$. The rate of diffusion, like the viscosity, was a function of pH. At pH 4.12 no measurable rate could be observed after 72 hours diffusion of a 0.3 per cent solution, indicating gel formation. At pH 7.5, the calculated mean diffusion constant was considerably lower than that of the de-

[2] There is little doubt, however, that structural viscosity may be detected at very low velocity gradients. Such measurements in a modified Couette apparatus are under way and will be reported elsewhere.

natured protein in 8 M urea. The calculated D_3 values varied widely from each other, probably due to restriction of free diffusion (Table I).

DISCUSSION

The denaturing effect of urea on pseudoglobulin is analogous to that observed for serum albumin. The apparent molecular asymmetry increases with increasing concentration of urea, while the molecular weight remains essentially unchanged. This latter finding is in agreement with the osmotic pressure measurements of Burk (6). The solutions of the denatured protein in urea are monodisperse, indicative of a uniform action of urea on all pseudoglobulin molecules.

The effects produced by guanidine hydrochloride are more complex. In 0.5 M concentration, guanidine produces only minor changes in apparent molecular shape and no changes in molecular weight. Diffusion measurements indicate, however, the presence of material of higher molecular weight. In 2 M solution, the apparent molecular asymmetry of the protein is markedly increased and the molecular weight is nearly twice that of the native protein.

When the guanidine hydrochloride concentration is increased to 3 M, the molecular asymmetry becomes drastically increased and about equal to that produced by 8 M urea solutions. The diffusion constant, however, does not decrease in proportion but, on the contrary, increases. The mean molecular weight, calculated from the limiting values of η_{sp}/c and D is about one-half of that of the native protein. Further increase in guanidine hydrochloride concentration, to 5.6 M, produces no further changes in apparent molecular shape or molecular weight except that the solutions now become monodisperse (Table I). The action of guanidine hydrochloride on pseudoglobulin is specific in that the molecule splits as it unfolds.[3] There does not appear to exist any dimensional relation between the denatured whole molecules and the denatured halves such as has been observed with the splitting of *native* protein molecules (10). Simultaneous splitting and unfolding has also been observed in the denaturation of myogen by urea (11).

The observed relation between the concentration of denaturing

[3] See (2) foot-note 8.

agent and the fraction of total protein irreversibly denatured after dialysis is in qualitative accord with the analogous relation found for serum albumin, and may be interpreted on the basis of the statistical considerations discussed previously (2). Quantitatively, however, these two sets of data differ from one another in that the limiting value of the fraction irreversibly denatured is 64 per cent for pseudoglobulin when denatured by urea and 84 per cent when denatured by guanidine hydrochloride, as compared with 15 per cent for serum albumin when denatured by either agent. This indicates fundamental differences in the intrinsic structure of these two proteins. The difference in maximum yield of irreversibly denatured pseudoglobulin produced by urea and guanidine hydrochloride is probably due to their different modes of action.

"Reversible" denaturation from 8 M urea solutions yields material of molecular size and shape similar to that of the native protein. Electrophoretic measurements on pseudoglobulin GI, "reversibly" denatured by 5 M urea, showed it to move with a single boundary on both the acid and alkaline side of the isoelectric point (12). The electrophoresis curves of the native and "reversibly" denatured material were practically indistinguishable from one another, except for differences in mobility.

The question of the true reversibility of denaturation demands a critical examination of the data at hand. It was shown that the protein remaining in solution after isoelectric precipitation of the irreversibly denatured fraction was polydisperse. However, the spread in diffusion constants was relatively narrow (3.8 to 4.6 $\times 10^{-7}$), suggesting that polydispersity was not due to incomplete separation of "reversed" denatured and denatured protein, but rather to a gradation in molecular size or shape of the "reversed" denatured protein itself. This assumption finds support in Polson's diffusion measurements on whole serum globulin (13) obtained by precipitation with half saturated ammonium sulfate, where the spread in calculated diffusion constants is comparable to that observed here for the unfractionated "reversed" denatured pseudoglobulin. Further support comes from the observed solubility properties. When "reversible" denaturation was carried out with pseudoglobulin GII, precipitation of the "reversibly" denatured protein was found to occur over the region of 1.1 and 1.6 M ammonium sulfate, whereas the salting-out region of the

native material was confined to between 1.36 and 1.6 M. Pseudoglobulin GI, when subjected to "reversible" denaturation, started to precipitate at an ammonium sulfate concentration of 0.8 M, as compared with 1.1 M for the native protein, and continued up to 1.36 M. One may conclude, that "reversible" denaturation did not result in the restoration of a distinct molecular configuration, but rather in the formation of molecular entities of related intrinsic structures. This conclusion is also in accord with the ideas expressed in a previous paper (12) concerning the continuous gradation in physical and chemical properties of the native globulins. A fraction approximating in properties the native material can be isolated from this mixture by subjecting it to fractional precipitation with salt under the same experimental conditions as have been used for the purification of the native protein. Further comparative studies of these fractions and of their immunological properties are under way.

The solubility properties of the irreversibly denatured protein are similar to those observed for the euglobulin fractions of normal horse serum as obtained by the method of isoelectric precipitation (14). Like these euglobulin components, the relative viscosity of this material is much higher than that of the native pseudoglobulin (15). Attempts to identify the protein in terms of molecular weight or shape were impeded by its anomalous behavior in respect to diffusion.

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SUMMARY

The denaturation of horse serum pseudoglobulin by urea follows a similar pattern to that observed for serum albumin. The apparent molecular asymmetry, determined by viscosity measurements, increases with increasing concentrations of urea, whereas the diffusion constants decrease in proportion. The molecular weight remains unchanged during denaturation.

The denaturing effects produced by guanidine hydrochloride depend on the concentration. 2 M guanidine hydrochloride appears to cause an aggregation of the protein molecules, the mean molecular weight being about twice that of the native protein. In

3 M solution the protein molecules split into halves as they unfold, whereas a further increase in the guanidine hydrochloride concentration, to 5.6 M, produces no additional changes in molecular size or shape except that the solutions become monodisperse.

Removal of the denaturing agent by dialysis causes a separation into two fractions which have been identified with "reversibly" and irreversibly denatured protein. The quantitative distribution between these two fractions is a function of the concentration of the denaturing agent. In equimolar concentrations, guanidine hydrochloride leaves a larger fraction irreversibly denatured than does urea.

The "reversibly" denatured protein, purified by fractional precipitation with ammonium sulfate, resembles, but is not identical with, the native protein in respect to molecular size, shape, and electrophoretic properties.

The irreversibly denatured protein resembles in respect to solubility and viscosity the euglobulin components as isolated by isoelectric precipitation from normal horse serum. The solutions exhibit a tendency for gel formation which is more pronounced on the acid side of the isoelectric point than on the alkaline side.

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A GRAVIMETRIC METHOD FOR THE DETERMINATION OF METHIONINE*

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With the recognition of methionine as an indispensable amino acid in nutrition (1) and the growing knowledge of the physiological function of methionine, particularly with reference to its ability to furnish methyl groups in the body, the estimation of this amino acid in food and tissue proteins assumes increasing importance. The present work, which deals with the development of a gravimetric method for methionine determination, was undertaken because it was found that not all protein preparations in which practical interest centers would readily yield to the existing methods. The new method depends upon the isolation of methionine sulfur as barium sulfate and was designed for the accurate analysis of both highly purified protein preparations and cruder protein materials isolated from common foods and tissues (2).

The methods hitherto available for methionine determination are those introduced by Baernstein (3–5). Modifications and refinements in technique, contributed by Kassell and Brand (6), have improved the degree of accuracy of the Baernstein methods. The new method depends upon the demethylation of methionine with concentrated hydriodic acid, resulting in the formation of homocysteine which, under the influence of the halogen acid, forms the homocysteine-thiolactone ring compound, a reaction outlined by Baernstein (4) and studied in detail by Riegel and du Vigneaud (7).

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† The data included in this paper are contained in a thesis submitted by D. Maxwell Teague in partial fulfilment of the requirements for the degree of Master of Science from the Department of Chemistry, Wayne University, Detroit (1941).

In boiling hydriodic acid, cystine is reduced to cysteine, which does not form a corresponding ring compound. In a mixture containing cysteine and homocysteine-thiolactone, only cysteine will react with cuprous oxide to precipitate an insoluble cuprous mercaptide; the thiolactone will not form a cuprous mercaptide because it does not contain a free sulfhydryl group. If the mixture is first treated with alkali to open the homocysteine-thiolactone ring and then, under suitable conditions, treated with cuprous oxide, the cuprous mercaptides of both cysteine and homocysteine are precipitated together. An analytical method based upon these reactions is here described.

Experiments with Homocystine

It was first necessary to determine whether, under suitable conditions, homocysteine can be quantitatively precipitated with cuprous oxide. Homocystine prepared by the method of Butz and du Vigneaud (8) and several times recrystallized was used in the experiments.

A 10 ml. aliquot containing 5.87 mg. of homocystine was made strongly alkaline by the addition of NaOH solution and allowed to stand 15 minutes,[1] then brought to neutrality with 20 per cent HCl, and a 2 ml. excess of HCl added. After reduction with zinc dust the solution was filtered, brought to pH 4 to 5 with sodium acetate, and the homocysteine precipitated at room temperature by addition of a water suspension of cuprous oxide. The precipitate was removed by centrifugation and a micro-Kjeldahl nitrogen determination made to estimate the homocystine recovery. In ten determinations an average recovery of 97.7 per cent of the original homocystine was obtained. Cystine solutions carried through the same procedure gave 99.1 per cent recoveries. Cystine and homocystine in the same solution were determined in a similar manner, the two cuprous mercaptides being precipitated together. Three experiments yielded an average recovery of 97 per cent of the amino acid nitrogen of the solutions, thus demonstrating that cysteine does not interfere with the homocysteine precipitation.

[1] The addition of alkali is not essential for the determination of homocystine, but it is necessary for the determination of the thiolactone yielded by the hydriodic acid digestion of proteins; therefore, the influence of alkali was tested in this procedure.

Method of Determining Methionine in Proteins

The procedure of the methionine method as it is applied to protein samples is described in detail. It is the result of extensive preliminary testing with synthetic methionine and methionine-cystine mixtures.

Hydrolysis of Protein Sample—A sample of protein (usually 0.5 to 1.0 gm.) containing approximately 6 mg. of methionine is placed in a 100 ml., round bottom flask; 25 ml. of concentrated hydriodic acid[2] are added and the mixture refluxed 18 hours. After cooling, the flask containing the hydrolysate is connected to a vacuum still and evaporated at about 40° under reduced pressure to a volume of 0.5 ml. Additions of 2.5 per cent HCl and repeated distillations serve to remove most of the iodine. The hydrolysate, which is light tan in color, is transferred to a 250 ml. centrifuge bottle and diluted to 50 ml. Moist silver chloride[3] is added (about 2 gm.) until an excess is present. The solution is vigorously shaken and, as the iodide is precipitated, liberated iodine turns the mixture darker brown. Further shaking with small additions of silver chloride insure complete removal of hydriodic acid. An excess of the reagent is not harmful. The precipitate is removed by centrifugation.

The supernatant is decanted through a filter and the precipitate washed with two 50 ml. portions of water. The combined, water-clear supernatants are then evaporated *in vacuo* to a syrup (about 2 ml.), which is washed into a 50 ml. volumetric flask and diluted to the mark. A 25 ml. aliquot (designated as Aliquot B) of the solution is removed with a pipette to a glass-stoppered centrifuge tube. The pipette is washed back into the volumetric flask with a few ml. of water. This solution (Aliquot A) is used for the deter-

[2] Merck's reagent quality, 55 per cent hydriodic acid of specific gravity 1.7 and *not* preserved with hypophosphite is satisfactory. Care must be taken that the HI used in hydrolysis is not more than dark red with iodine. If extensive decomposition of the unpreserved HI has taken place, it is advisable to redistil before use. All operations with hydriodic acid should be carried out in glass equipment.

[3] A suitable silver chloride preparation is made by adding dilute HCl to a saturated solution of silver lactate. The silver chloride precipitate is washed several times with distilled water by decantation and stored in the dark under a layer of water to keep it moist.

mination of cystine sulfur alone. Aliquot B is used for the determination of cystine plus methionine sulfur.

Determination of Cystine in Aliquot A—The procedure for the determination of cystine is adapted from the procedure of Graff, Maculla, and Graff (9). To Aliquot A are added 1 ml. of 20 per cent HCl and 300 mg. of zinc dust and the mixture is allowed to stand at room temperature for 2 hours. The zinc is then removed by filtration. The filtrate is brought to pH 4 to 5 by the dropwise addition of saturated sodium acetate during rapid mechanical stirring.[4]

A suspension of finely divided, bright red cuprous oxide[5] is added dropwise to the sample during mechanical stirring. The light gray, flocculent precipitate of cysteine cuprous mercaptide, which forms almost immediately, acquires a red color as an excess of cuprous oxide is added. Cuprous oxide is added until a small but definite excess is present and the solution is stirred for about 30 seconds to insure complete reaction with the cysteine. The precipitate is collected by centrifugation and washed three times with 30 ml. of a citrate-acetate buffer solution[6] to remove sulfur-containing contaminants. A microgravimetric sulfur determination serves for the estimation of the cysteine content of the precipitate.

Determination of Cystine and Methionine in Aliquot B—The solution is brought to neutrality by the addition of 5 N NaOH, after which an excess of 1 ml. is added. 15 minutes at this alkaline pH suffice to open the thiolactone ring but do not destroy cystine. The sample is acidified with 2 ml. of 20 per cent HCl, 300 mg. of zinc dust are added, and the centrifuge tube is loosely stoppered. The solution is allowed to stand overnight at room temperature and is then heated for 2 hours in a steam bath. The acidity of the solution is now sufficiently close to that required for the cuprous

[4] Universal pH indicator paper (Eberbach) or Congo red paper is satisfactory.

[5] The cuprous oxide reagent should contain a minimum amount of sulfur. Baker's, C.P., cuprous oxide, red powder has the desired properties; however, the dry oxide should be wet by shaking with water and ground in a mortar until thoroughly suspended.

[6] The stable buffer stock solution is made by dissolving 12 gm. of sodium citrate and 15 gm. of citric acid in 200 ml. of water and adding 20 ml. of glacial acetic acid. The solution is diluted to 10 volumes before use.

oxide reaction, so that no further adjustment of pH is necessary. The solution is quickly decanted from the excess zinc into a centrifuge tube containing a few drops of the cuprous oxide-water suspension. The zinc remaining in the bottom of the tube is washed with water and the washings decanted through a small filter. This warm (60°) sample, containing excess cuprous oxide, is stirred rapidly for 15 seconds, immediately stoppered, and centrifuged for 1 minute. The supernatant is removed by decantation.

Inasmuch as homocysteine yields a cuprous mercaptide only when in its sulfhydryl form and, in contrast to cysteine, is susceptible to oxidation by air even when in an acid medium, it is essential that the operations subsequent to the final treatment with zinc be carried out rapidly and with minimum exposure to air.

The precipitate obtained from Aliquot B consists of a mixture of the cuprous mercaptides of cysteine and homocysteine. A microgravimetric determination serves for the estimation of the cysteine plus homocysteine sulfur content of the mixed mercaptides. Washing the precipitate with buffer is not recommended, inasmuch as the presence of sulfur contaminants is quite unlikely.

For the sulfur analysis of the cuprous mercaptide precipitates from Aliquots A and B, a micro adaptation of the Denis method (10) is satisfactory.[7] This may be applied after the precipitate is dissolved in 1.5 ml. of concentrated HNO_3.

Calculation and Correction of Methionine Values—Some losses of methionine occur during the hydriodic acid hydrolysis procedure. This has been observed both by Baernstein and by Kassell and Brand (6) in their determinations of the homocysteine residue. The correction factor to be used with the foregoing procedure was determined by analyzing pure methionine samples. Analyses of ten samples of pure methionine in solution (6 to 12 mg.) yielded recoveries of 86.9 to 91.6 per cent, with an average of 90.1 per cent. A correction factor of 1.11, therefore, was employed.

[7] Satisfactory combustion is obtained with 1 ml. of the Denis reagent. The resulting mixture is dissolved in 5 ml. of 10 per cent HCl, filtered, and the $BaSO_4$ precipitated from a volume not exceeding 40 ml. The precipitate is filtered in a weighed micro crucible conveniently prepared by cutting a Corning Glass Works, Pyrex sinter glass filter stick of finest porosity (diameter 13 mm.) 5 mm. below and 30 mm. above the sinter glass insert.

1 mg. of $BaSO_4$ is equivalent to 0.515 mg. of cystine, or 0.709 mg. of methionine (corrected). The methionine content of a protein sample may be calculated as the difference in sulfur content of the cuprous mercaptides obtained in Aliquots A and B:

$$\% \text{ methionine in protein sample (corrected)} =$$

$$\frac{\text{mg. } BaSO_4 \text{ from B minus mg. } BaSO_4 \text{ from A}}{\text{gm. of sample}} \times 0.142$$

Triplicate analyses of a protein for methionine content can be accomplished in 2 days working time. Replicate analyses have been found to be in good agreement for proteins ranging in methionine content from 0.57 to 4.09 per cent.

The same procedure has been applied to mixtures of equal quantities of cystine and methionine. Methionine analyses of seven mixtures yielded recoveries of 96 to 103 per cent when the correction factor was applied. The cystine recoveries were sometimes low and ranged from 80 to 100 per cent of the amount originally present.

Methionine Content of Some Protein Materials—The new method of analysis was extended to an investigation of several common materials: arachin, casein, edestin, commercial egg white, gelatin, human globin, lactalbumin, and beef muscle. Table I presents the composition of these substances with respect to total nitrogen, total sulfur determined after combustion in the Parr oxygen bomb, and the distribution of total sulfur as methionine sulfur, sulfate sulfur, and cystine sulfur determined by the method of Graff, Maculla, and Graff (9). The sulfate sulfur was determined gravimetrically as $BaSO_4$ after 6 hours hydrolysis of the protein materials with 20 per cent HCl. Sulfate sulfur constitutes an appreciable fraction of the total sulfur of some of the protein preparations.

94 per cent or more of the total sulfur of the proteins could be accounted for by this system of analysis, except in the two vegetable proteins, edestin and arachin. The total nitrogen contents demonstrate that the protein materials used in the experiment were not contaminated to any great extent with non-protein substances. In Table II the results obtained with proteins by the present method of methionine determination are compared with those determined by other workers with other methods of analysis.

TABLE I

*Sulfur Partition of Proteins**

Protein	Total N	Total S†	S partition			Total S recovered‡
			Cystine S	Methionine S	Sulfate S	
	gm. per 100 gm.	*gm. per 100 gm.*	*gm. per 100 gm.*	*gm. per 100 gm.*	*gm. per 100 gm.*	*per cent*
Arachin	17.7	0.474	0.232	0.123	0.058	87.1
Casein	15.81	0.843	0.088	0.671	0.058	96.9
Edestin	18.45	0.944	0.323	0.494	0.033	90.0
Egg white	14.98	1.816	0.809	0.879	0.026	94.4
Gelatin	18.35	0.411	0.005	0.175	0.207	94.2
Globin (human)	16.66	0.681	0.323	0.328	0.013	97.5
Lactalbumin I	15.7	1.546	0.798	0.641	0.069	97.5
" II	15.7	1.547	0.876	0.639	0.040	100.5
Muscle (beef)	14.83	1.051	0.272	0.691	0.022	93.7

* Corrected for moisture and ash.

† Determined by the oxygen bomb method.

‡ Sum of cystine, methionine, and sulfate sulfur in per cent of total sulfur, as determined with the oxygen bomb.

TABLE II

Comparison of Percentage Methionine Values for Proteins

Protein	Values by gravimetric method	Kassell and Brand (6, 11)	Baernstein (5)	Baernstein (4, 12)	Birkofer and Taurins (13)	Other bibliographic values
Arachin	0.57			0.54*		0.77 (14)
Casein	3.12	3.17†	3.31†	3.50†		3.03 (15)
		3.06‡	3.10‡	3.53*		
Edestin	2.30	2.4†	2.38†	2.39†		2.3 (16)
		2.3‡	2.20‡	2.07*		
Ovalbumin		5.22†	5.07†	5.24†		4.85 (15)
		5.10‡	4.49‡	4.57*		
Egg white	4.09			5.10†		
				5.29*		
Gelatin	0.81					
Globin, beef					1.70–1.89	
" horse					0.89–1.03	0.94 (15)
" human	1.52				0.36–1.47	
Lactalbumin	2.98	2.81†	2.45†	2.62†		
	2.97	2.89‡	2.32‡	2.63*		
Muscle, beef	3.21			3.66*		

* Volatile iodide (alcoholic $AgNO_3$ absorbent) (12).

† Volatile iodide.

‡ Homocysteine titration.

The methionine content of gelatin, 0.81 per cent, has not previously appeared in the literature. Substantial agreement with values determined by others gives additional proof of the validity of the new method.

SUMMARY

A gravimetric method for methionine determination, involving the isolation of methionine sulfur as $BaSO_4$, has been outlined.

The method has been tested by applying it to pure methionine and homocystine, to methionine and cystine mixtures, and to eight common protein materials.

The cystine and methionine contents of arachin, casein, edestin, egg white, gelatin, human globin, lactalbumin, and beef muscle have been determined.

The sulfur partitions of these eight protein materials are presented.

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ISOLATION OF AN ANTIBIOTIN FACTOR FROM EGG WHITE

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Eakin, Snell, and Williams (1, 2) have shown that it is possible to prepare highly active concentrates from raw egg white which combine stoichiometrically with biotin and render it unavailable to yeast. At the time the preliminary note by Eakin *et al.* (1) appeared, it had been observed in this laboratory that egg white rendered biotin unavailable for *Clostridium butylicum* and the concentration of the injurious factor in egg white was begun, since it appeared that such a reagent would be useful in demonstrating the biotin needs of the more fastidious microorganisms (3). A substance was obtained which was homogeneous on electrophoresis and in the ultracentrifuge. During the writing of this manuscript, the second paper by Eakin and his associates (2) appeared in which they report a further concentration of the active ingredient of egg white. Although their method of purification differs somewhat from that used by us, their latest material has essentially the same biological activity as ours. Consequently, this paper confirms and, in several respects, extends their work. In particular, experimental evidence is presented as to the purity of our material.

Eakin *et al.* have named their material *avidin*. In this paper we refer to our substance as an antibiotin factor. This term may be abbreviated as AB and the compound which AB forms with biotin as BAB. Since results obtained thus far in this laboratory indicate that the phenomenon of antivitamins extends to other members of the B group, a uniform nomenclature may serve to clarify the field. Thus a substance active against thiamine, *i.e.* antithiamine, could be abbreviated AT, and the inactive complex TAT.

EXPERIMENTAL

Method of Assay—A basal medium was made by dissolving the following substances in 1 liter of water: glucose 200 gm., KH_2PO_4 8 gm., NH_4NO_3 18 gm., $CaCl_2 \cdot 6H_2O$ 1.3 gm., thiamine 1 mg., riboflavin 2 mg., pantothenic acid 2 mg., nicotinic acid 2 mg., adenine 25 mg., uracil 5 mg., pyridoxine 2 mg., pimelic acid 2 mg., choline chloride 50 mg., and inositol 200 mg. 5 cc. portions of this solution were placed in 50 cc. Erlenmeyer flasks and enough water was added so that when the dilutions of the AB preparation to be assayed were added the volume would be 10 cc. Sufficient biotin methyl ester[1] was also included to give a final concentration of this material of 0.001 γ per cc.; the flasks were stoppered loosely with cotton and heated in an autoclave (15 pounds for 15 minutes). The proper dilutions of the preparation to be tested were then added, and each flask was inoculated[2] as described elsewhere (4). After the flasks had been incubated at 30° for 24 hours, the turbidity of the contents of each was determined quantitatively (4). The turbidity was plotted against the quantity of preparation added per cc., and the amount required to produce half maximal inhibition of growth was ascertained. This amount was said to contain an antibiotin factor unit (ABU). Egg white contained 1 ABU in 1.2 to 1.5 mg.; that is, 1.2 to 1.5 mg. of egg white combined with 0.001/2 or 0.0005 γ of biotin methyl ester.

Preparation of Antibiotin Factor—1 volume of fresh egg white was slowly added to 4 volumes of acetone which was stirred vigorously. After standing for several hours, the mixture was filtered in a press, the filter cake was ground in a meat chopper and placed in a volume of water one-third that of the original egg white, and the suspension again filtered in a press. The extract was discarded. The cake was ground and extracted with 1 per cent NaCl solution

[1] We wish to thank Dr. Vincent du Vigneaud for gifts of crystalline biotin methyl ester.

[2] Although yeast growth was inhibited by AB which was introduced before inoculation, when the AB was added 6 hours after inoculation (growth just barely visible), no effect was observed on the subsequent rate of growth. This suggested that the biotin of the medium was trapped in the yeast cells and passed along to the new buds internally, and that the yeast cell was impervious to AB.

equal in volume to that of the original egg white. The suspension was filtered in a press and the cake was again extracted with salt solution. The combined extracts, which contained the antibiotin factor, were filtered through paper and enough solid ammonium sulfate was added to the filtrate to half saturate it. The precipitate was filtered off and discarded, and the filtrate was treated with enough ammonium sulfate to saturate it completely. The precipitate was filtered off, washed, and dissolved in water. This solution was dialyzed against running tap water for 30 hours. The precipitate which formed was collected by centrifugation and washed repeatedly with water. The inactive filtrate was discarded. The precipitate was extracted with half saturated ammonium sulfate solution whose volume was one-tenth that of the original egg white, and the residue was filtered off and washed. The filtrate and washings were then dialyzed against running water for 30 hours. The precipitate which formed was collected in a centrifuge, and washed with water. In most instances it was dried at −40° to a fluffy white product.

The material obtained in this manner varied in potency from one preparation to another; namely, from 5 to 10 ABU per microgram. 40 to 80 per cent of the activity of egg white was recovered in such preparations. Electrophoretic examination showed that those preparations with the highest potency consisted of essentially one homogeneous component, whereas the others contained a second inert constituent which, however, could be readily eliminated by electrophoretic separation. Our purest material was therefore obtained with the latter procedure and always contained 10 ABU per microgram.

The methods of electrophoretic analysis and separation used in this research have already been described (5). The solid was suspended in one of the buffer solutions listed in Table I and dialyzed against the buffer for several days. The undissolved matter was centrifuged out and the clear solution was examined at 0°. Portions of the solutions of the electrophoretically separated components were assayed for potency. As may be deduced from the mobility data of Table I, the active material migrated toward the cathode at all pH values below 10.0, whereas the accompanying impurity, when it was present, migrated cathodically

at pH values below 4.3 and anodically above this value. Neither of these components was identical with any of the previously recognized constituents of egg white (5).

Properties of Antibiotin Factor—Many of the physical properties of AB are apparent from the manner of preparation. Thus it is insoluble in water and in saturated ammonium sulfate solution, sparingly soluble in dilute salt solutions, and rather soluble in strong salt solutions.

The preparations obtained were not obviously crystalline. As the protein precipitated during dialysis, it appeared as very small

TABLE I

Electrophoretic Mobilities of Components of Antibiotin Factor Preparation

Buffer solvent	pH at 25°	$U \times 10^5$ at 0°	
		AB	Impurity
0.1 N HCl	1.09	8.9	7.9
0.02 " NaAc,* 0.2 N HAc, 0.08 N NaCl	3.61	6.4	2.3
0.1 N NaAc, 0.02 N HAc ..	5 35	4.7	−3.0
0.02 " NaV,† 0.02 " HV, 0.08 N NaCl	7.84	2.2	−6.8
0.1 N NaOH, 0.12 N glycine	10 35	−0.3	−9.0

* Ac = acetate.

† V = diethyl barbiturate.

white particles of uniform shape and size but without definite crystal faces.

Combustion analyses showed the presence of C 45.5, H 6.6, N (Dumas) 10.8, ash 10.6. Analysis of one sample revealed 1.6 per cent phosphorous.

A solution of the antibiotin factor was very kindly examined for us in the ultracentrifuge by Dr. A. Rothen of these Laboratories. It was homogeneous and had a sedimentation constant, referred to water, of 4.7×10^{-13} at 20°. An accurate value for the molecular weight was not obtained, since the diffusion constant was not determined. If the particles are assumed to be spherical, the molecular weight would be close to 70,000, whereas it would be somewhat less than this if the molecules were not spherical.

The mobility-pH curve of the antibiotin factor was essentially straight over the pH interval studied. In order to determine whether combination with biotin affected the mobility, a solution of pure AB was added to an excess of biotin methyl ester and the mixture was dialyzed. Examination of the physiologically inactive BAB complex at pH 3.61 revealed that its mobility, 6.37 $\times 10^{-5}$, was not significantly different from that, 6.45 $\times 10^{-5}$, of AB. This result indicated that the size and charge of the antibiotin factor were not changed appreciably on combination with biotin and was thus consistent with our conclusion (*vide infra*) that only 1 molecule of biotin methyl ester (mol. wt., 256) combined with each molecule of AB (mol. wt. $\leq$ 70,000).

The antibiotin factor is relatively stable toward changes of pH and temperature. Activity was retained when solutions of AB in dilute acetic acid were boiled. No appreciable loss of potency was observed after solutions of AB had stood at room temperature for several days or in the ice box for several weeks at pH ranging from 1.0 to 11.0. The antibiotin factor was precipitated from acid aqueous solution by picric acid and by flavianic acid, and activity could be regained from such precipitates by treating them with alcoholic ammonia.

Attempts were made to discover whether AB possessed enzyme activity. No proteinase, antitrypsin, or lysozyme activity could be demonstrated.

Specificity of Antibiotin Factor—The recognition of the high isoelectric point of AB led to the assay of other basic proteins for AB activity. Salmine had 0.2 per cent of the activity of AB; *i.e.*, 0.02 ABU per microgram. A nucleohistone prepared from liver by Dr. A. E. Mirsky of the Hospital of the Rockefeller Institute, had 0.1 per cent of the activity of AB. The inhibitory action of both of these proteins of widely separated origin was erased by additional amounts of biotin. It may be that these substances were impure and contained the same AB as hen's egg. If this is true, the presence of AB in fish and in mammals is thereby indicated. If the activity is an attribute of the pure proteins, it is difficult to envision the combination, for the low activity would require that several molecules of protein combine with 1 molecule of biotin.

DISCUSSION

The above data demonstrate that it is possible to prepare from hen's eggs a substance which is approximately 15,000 times more effective in inactivating biotin than is the egg white itself. Although not obviously crystalline, this preparation was homogeneous in the electrophoresis apparatus and in the ultracentrifuge. On the basis of these two criteria it was, therefore, a pure substance. The data regarding molecular size indicate that 1 molecule of biotin combined with 1 of the active protein. This follows from the fact that their combining weights are, roughly, in the same ratio, *i.e.* 0.005:1, as their molecular weights, *i.e.* 256:70,000. It must be remembered that the values obtained by assay involved the inherent errors of a biological test and that the true molecular weight of AB may be somewhat less than the value computed from its sedimentation constant alone.

SUMMARY

An antibiotin factor, a basic protein (isoelectric point pH 10) which combines firmly with biotin, has been isolated from egg white. The preparations were 15,000 times more active than egg white and were homogeneous in electrophoresis and sedimentation experiments. Some chemical and biological properties of the protein have been investigated. This protein is similar in biological activity to the substance isolated from egg white by Eakin, Snell, and Williams (1) and called by them avidin.

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THE ACTION OF HIPPURICASE ON RING-SUBSTITUTED DERIVATIVES OF HIPPURIC ACID*

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Hippuricase, originally called histozyme (1), is an enzyme which promotes the hydrolysis, at the peptide linkage, of hippuric acid. It is widely distributed in animal tissues (2–5), molds (6–9), and bacteria (10, 11). Its differentiation from other enzymes known to attack peptide linkages is clarified by the inability of trypsin (12, 13), pancreatin (14), or erepsin (13) to hydrolyze hippuric acid. The failure of the ordinary digestive enzymes to attack hippuric acid has been further demonstrated by Griffith and Cappel (15), who found that hippuric acid was not destroyed in any part of the alimentary tract except the large intestine. The activity there was attributed to bacteria.

The data presented in this paper are the relative rates of hydrolysis (with hippuricase) of hippuric acid and various ring-substituted derivatives. We attempt to show the influence of the group and the position of substitution. Studies with related compounds are reported to define more accurately the specificity of the enzyme.

EXPERIMENTAL

The substituted hippuric acids were prepared by converting the corresponding substituted benzoic acids to the acid chlorides by phosphorus pentachloride, and then treating the acid chlorides with a concentrated solution of glycine by the Schotten-Baumann technique.

The purity of the products was checked by their melting points, with reference for the methyl derivatives to the values given by

* The material contained in this paper is part of a dissertation submitted by Sydney Ellis in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Boston University Graduate School.

Gleditsch and Moeller (16), for the halogen derivatives by Novello, Miriam, and Sherwin (17), and for the nitro derivatives by Jaffe (18).

Taka-diastase is known to be a source of hippuricase (19). Preliminary tests of a preparation[1] demonstrated that a 4 gm. per cent solution possessed sufficient hippuricase activity for the present work. The pH of a 4 gm. per cent solution was found to be 5.8; such a solution has considerable buffer capacity: when mixed with the substrates as described below, the pH varied only between 5.8 and 5.4 in all experiments. No further pH control was attempted. The rate of hydrolysis of a 0.02 M solution of sodium hippurate was found to be proportional to the concentration of the enzyme. With varying concentrations of hippurate (0.02 to 0.1 M) in a 4 gm. per cent solution of taka-diastase, the absolute amount of hippurate hydrolyzed in a given time was the same. The kinetics of hydrolysis in the range of enzyme and substrate concentrations used are therefore of zero order.

On the basis of the preliminary studies just described, the following standard procedure was followed in all subsequent experiments. 0.0005 mole of the substrate was weighed into rubber-stoppered tubes graduated at 25 cc., dissolved in an equivalent volume of 0.1 N sodium hydroxide, mixed with 10 cc. of a 10 gm. per cent solution of taka-diastase (which was previously prepared and incubated 1 hour at 37° before use), made up to 25 cc. with water at 37°, and covered with 1 cc. of xylene. The tube was stoppered, shaken, and suspended in a thermostat at 37°.

Sørensen formol titrations were used in following the course of the hydrolysis. Control solutions, containing no substrate, were incubated simultaneously. The amount of standard base used in titrating 5 cc. of the test solutions (less the amount used in titrating 5 cc. of the control solution) was in each case converted to percentage hydrolysis; the results are shown in Fig. 1.

A comparison of the curves of the substituted hippuric acids with that of hippuric acid makes it evident that substituents in the ortho position exert a strong inhibitory effect upon the hydrolysis; with substituents in the meta position there is a consistent

[1] The preparation of taka-diastase was supplied by the courtesy of Parke, Davis and Company, and was stated to liquefy 450 times its weight of starch in 10 minutes.

acceleration of the rate of hydrolysis; substituents in the para position appear to exert but a negligible effect upon the rate. The constitution of the substituting group seems to have little effect as compared with the marked effect of its position.

For further light upon the mechanism whereby the substituents

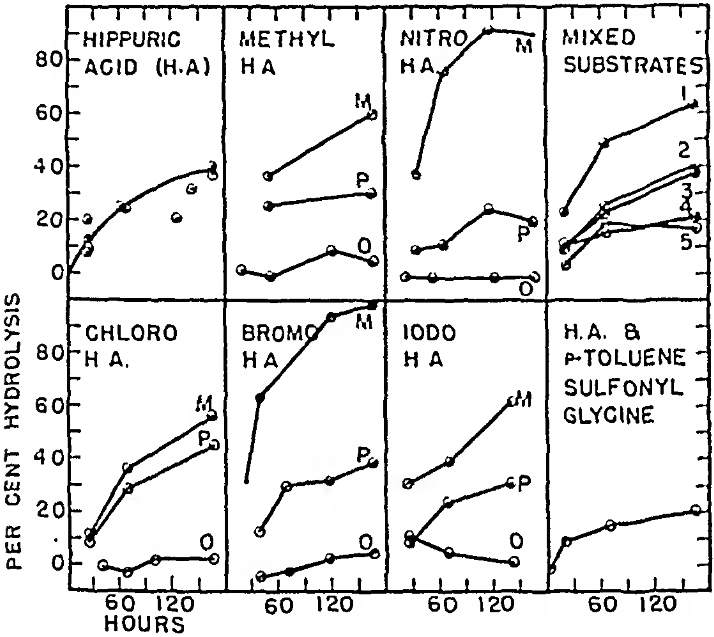

FIG. 1. Rates of hydrolysis (in the presence of hippuricase) of hippuric acid and of certain derivatives and related compounds. The numbers on the chart marked "mixed substrates" have the following significance: Curve 1, hippuric acid and *m*-iodohippuric acid; Curve 2, hippuric acid; Curve 3, hippuric acid and *p*-iodohippuric acid; Curve 4, hippuric acid and *o*-chlorohippuric acid; Curve 5, hippuric acid and *o*-iodohippuric acid. All the experiments represented were carried out according to the standard procedure described in the text.

alter the hydrolytic rate, another series of experiments was carried out with the same standard procedure and the following substrates: hippuric acid 0.02 M; hippuric acid 0.01 M and *o*-chlorohippuric acid 0.01 M; hippuric acid 0.01 M and *o*-iodohippuric acid 0.01 M; hippuric acid 0.01 M and *m*-iodohippuric acid 0.01 M; hip-

puric acid 0.01 M and *p*-iodohippuric acid 0.01 M. The graph of the results (labeled "mixed substrates") shows that mixed substrate containing the para isomer hydrolyzed at the same rate as the simple hippuric acid, the mixed substrate containing the meta derivative hydrolyzed more rapidly, while those containing ortho-substituted derivatives were inhibited to about one-half the rate with hippuric acid alone. We believe these results to indicate that groups in the ortho position do not inhibit the formation of the enzyme-substrate complex, but rather, once the complex is formed, fail to permit the breaking of the peptide bond. Formation of the complex is shown by the decreased rate of hydrolysis of the unsubstituted hippuric acid, demonstrating a reduction in the concentration of free enzyme on account of complex formation.

Since *p*-toluenesulfonylglycine was found to be resistant to hippuricase, a mixture of 0.01 M hippuric acid and 0.01 M *p*-toluenesulfonylglycine was tested as a substrate and found to be hydrolyzed at the same rate as the mixed substrate containing ortho-substituted derivatives. Since *p*-toluenesulfonylglycine and ortho-substituted hippuric acids are not hydrolyzed by hippuricase, we must assume that the hydrolysis in these mixtures was a measure of the hydrolysis of hippuric acid alone. Again, the most acceptable explanation of the slower rate is the combination (reversibly) of the unhydrolyzable compounds with the enzyme. This last experiment also indicates that the α-amino acid radical is the important group in the enzyme-substrate combination.

In the course of the work a number of other compounds were tested to obtain more information about the specificity of hippuricase. The recently emphasized importance of acetylization led to the testing of several acetylamido compounds. Acetanilide, acetophenetidin, and the three isomeric acetotoluides were found to be unattacked by the enzyme. Since these compounds are but slightly soluble, a related series which could be treated in the same manner as hippuric acid was studied—the acetylamidobenzoic acids. They were not hydrolyzed. Since acetylglycine (aceturic acid) is hydrolyzed (20, 21), these results also indicate the importance of the glycine portion.

Benzamide is not hydrolyzed by hippuricase (22); we confirmed this finding and tested other soluble compounds having carbamyl groups. Acetamide, urethane, and urea were not hydrolyzed.

The guanido group of creatine was not attacked. There was no hydrolysis of benzanilide.

We found that benzenesulfonylglycine was not hydrolyzed by hippuricase. Mazza and Pannain (23) reported rapid hydrolysis of *p*-toluenesulfonylglycine by a tissue extract claimed to contain hippuricase. We were able to verify the accuracy of the formol titration of amino groups in the presence of benzenesulfonate ions by titrating known concentrations of glycine in the presence of 1 per cent sodium benzenesulfonate. *p*-Toluenesulfonylglycine was prepared and tested with the enzyme. Our hippuricase preparation did not hydrolyze this compound and does not appear to hydrolyze the sulfonamide linkage.

The ability of this enzyme to hydrolyze glycocholic acid and not taurocholic acid (another indication of the significance of the glycine radical in its specificity) was reported by Grassmann and Basu (12). We found that glycocholic acid was hydrolyzed about 10 per cent in 7 days under our conditions, while the hydrolysis of taurocholic acid in the same period was insignificant.

DISCUSSION

This work correlates with experiments, which have been done over a period of many years, dealing with the excretion of hippuric acid and its derivatives by animals fed benzoic acid (or substances convertible to benzoic acid) and its derivatives. Quick (24) has given a summary of this work and has added significant quantitative studies. His results show insignificant formation of ortho-substituted hippuric acids. In his series of chloro, bromo, and nitro derivatives, excretion of the meta-substituted hippuric acids was significantly greater than that of the para isomers. On the other hand *p*-toluylglycine was excreted to a greater extent than *m*-toluylglycine.

Our present knowledge of the specificity of hippuricase can be summarized in a few general statements. This enzyme catalyzes the hydrolysis of benzoylated α-amino acids, preferentially the natural optical isomer (14). Benzoylated β-amino acids are not affected (25, 26). An α-hydrogen must be present in the amino acid; benzoyl-α-aminoisobutyric acid is not hydrolyzed (26). The hydrogen on the nitrogen of the peptide linkage is considered necessary (25). Mazza and Pannain (23) contradict this, but

report action by the enzyme on several compounds with which others have found it inactive. Our own findings are summarized below. For a general review of the subject of hippuricase, the paper of Trolle (27) should be consulted.

SUMMARY

1. In the presence of taka-diastase as a source of hippuricase, the relative rates of hydrolysis of the isomeric mono-substituted (chloro, bromo, iodo, nitro, and methyl) hippuric acids were studied.

2. Ortho substitution of the above groups was found to inhibit hydrolysis, meta substitution to accelerate hydrolysis, and para substitution to have a negligible effect.

3. Evidence is offered that ortho substitution does not prevent combination with the enzyme, but prevents the breaking of the peptide linkage.

4. Evidence, in addition to that in the literature, is offered that the combination of the enzyme with the substrate takes place through the glycine (or amino acid) portion.

5. The following substances are not hydrolyzed by the action of hippuricase: acetanilide, acetophenetidin, the acetotoluides, the acetamidobenzoic acids, benzamide, acetamide, urethane, urea, creatine, benzanilide, benzenesulfonylglycine, *p*-toluenesulfonylglycine.

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THE THREONINE, SERINE, CYSTINE, AND METHIONINE CONTENT OF PEANUT PROTEINS*

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An extensive peanut breeding program has been in progress at this Station for several years. Since the earlier work of Johns, Jones, and others (1–6) on peanut proteins, some new amino acids have been discovered and improved methods of determination have appeared in the literature. An investigation of the composition of peanut proteins has been started in order to determine whether the proteins of the new strains are similar in composition to those reported for other varieties and in order to obtain more complete data on the amino acid composition of arachin and conarachin.

EXPERIMENTAL

Sound, clean, shelled peanuts of Strain 18-14 (Pearl × North Carolina Runner) were pressed in a hydraulic, steel cage press to remove most of the oil. The seed-coats were removed, and the kernels ground finely and extracted with petroleum ether. The oil-free meal contained 44.9 per cent crude protein (N × 5.5).

Arachin and conarachin, prepared according to the method of Johns and Jones (1), were dialyzed, with a No. 300 cellophane membrane, until the chlorides were removed. The arachin and conarachin contained 0.64 and 0.40 per cent ash and, on an ash-free basis, 17.97 and 18.00 per cent nitrogen and 0.52 and 1.22 per cent sulfur, respectively.

The nitrogen distribution was determined by Cavett's modification (7) of the Van Slyke method. The results were similar to those obtained by Johns and Jones (2) for arachin and conarachin.

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Threonine and serine were determined by the methods of Shinn and Nicolet (8, 9), which are based on the determination of the acetaldehyde and formaldehyde formed by the reaction of periodic acid with these amino acids. Excellent recoveries were obtained on analysis of known mixtures of these compounds with other amino acids and ammonium chloride. Total β-hydroxy-α-amino acids were determined by the periodate method of Van Slyke,[1] except that the ammonia was absorbed in boric acid solution and titrated with N/70 hydrochloric acid, with a mixed indicator containing methyl red and tetrabromophenol blue. Excellent recoveries were obtained with this method also.

TABLE I

β-Hydroxy-α-amino Acids of Peanut Proteins

Protein	Threonine	Threonine as serine equivalent	Serine	Total serine equivalent	Total hydroxyamino acids, by NH_3 evolution, as serine equivalent	Other hydroxyamino acids, serine equivalent
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Arachin........	2.56	2.26	5.20	7.46	7.51	0.05
Conarachin....	2.02	1.78	4.99	6.77	7.78	1.01

TABLE II

Sulfur Distribution of Peanut Proteins

Protein	Cystine + cysteine S	Methionine S	Sulfate S as H_2S	Total S	
				(1 + 2 + 3)	Found (Pregl)
	(1)	(2)	(3)	(4)	(5)
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Arachin..................	0.40	0.14	0.02	0.56	0.52
Conarachin.............	0.78	0.45	0.00	1.23	1.22

The results for threonine, serine, and total β-hydroxy-α-amino acids are shown in Table I.

Cystine plus cysteine and methionine were determined by the methods of Baernstein (10) with the modifications introduced by Kassell and Brand (11). Sulfur was determined by Saschek's modification (12) of the Pregl method, except that the barium sulfate was ignited.

[1] Van Slyke, D. D., personal communication.

The results for arachin were 1.51 per cent cystine and 0.67 per cent methionine. Those for conarachin were 2.92 per cent cystine and 2.12 per cent methionine. The sulfur distribution is shown in Table II.

DISCUSSION

Martin and Synge (13) reported that 1.1 per cent of the total nitrogen of a sample of total globulin of the peanut was present as threonine nitrogen. Since they did not give the nitrogen content, and their recovery of acetaldehyde formed from threonine was poor, it is not possible to compare their results with those given here.

Baernstein (6) found 0.54 per cent methionine and 1.33 per cent cystine in a sample of arachin which contained 0.42 per cent sulfur, compared with 0.67 per cent methionine and 1.51 per cent cystine in a sample containing 0.52 per cent sulfur reported here. The total sulfur recovered by Baernstein was 111.9 per cent, compared with the present value of 103.8 per cent. While methionine has not been reported heretofore for conarachin, Jones *et al.* (4) found 3.00 per cent cystine compared with the value of 2.92 per cent cystine reported here.

SUMMARY

Arachin and conarachin were prepared from peanuts, purified, and analyzed for threonine, serine, cystine, and methionine.

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CYTOCHROME C PEROXIDASE

II. THE PEROXIDASE-HYDROGEN PEROXIDE COMPLEX

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(Received for publication, September 29, 1941)

In a previous publication (1) we reported the isolation, purification, and properties of a new enzyme, cytochrome *c* peroxidase. Since the publication of that report we have carried out further experiments on this enzyme, particularly with a view toward establishing its capacity to combine with hydrogen peroxide. In the course of these experiments we have found it expedient to modify the formerly reported procedure for isolation and purification and have been able to obtain a product which has a 3-fold greater purity than the best product first reported. The result of this extended work is the subject of this paper.

Test

The spectrophotometric test described in the previous paper (1) was also used in this work to determine the enzyme activity. Cytochrome *c* dissolved in 0.02 M phosphate buffer is reduced with hydrogen and palladium asbestos, the final concentration of reduced cytochrome *c* being 1.5×10^{-8} mole per cc. In the course of the reduction, hydrogen peroxide is formed from the dissolved O_2. The concentration of the hydrogen peroxide thus formed is 4.5×10^{-8} mole per cc., a 6-fold excess over that of the cytochrome *c*. The activity of an enzyme preparation is determined by observing the rate of oxidation of reduced cytochrome *c* by the

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hydrogen peroxide in the presence of a given amount of enzyme. It was found that this reaction obeys the kinetic equation,

$$-\frac{d \log (\mathrm{CyFe}^{++})}{dt} = K(E)$$

where (CyFe^{++}) is the concentration of the reduced cytochrome c and (E) represents the concentration of the cytochrome c peroxidase. The cytochrome c peroxidase unit of activity is defined as that amount of peroxidase which gives a value of 1 min.^{-1} for $-d \log (\mathrm{CyFe}^{++})/dt$. The relative purity of any preparation is determined by the number of enzyme units per mg. of protein and is denoted by the symbol, Q.

Isolation and Purification

Several modifications were made in the procedure previously used in obtaining cytochrome peroxidase. As a result, a higher degree of purification was obtained.

Principle—Washed and dried bakers' yeast is ground and allowed to autolyze in water saturated with toluene. The presence of toluene alone increases the yield 5-fold and the purity of the resulting extract about 4-fold. This action of toluene is probably due both to its ability to break cells, thus speeding up autolysis, and to its bactericidal action which prevents bacterial destruction of the enzyme during the long autolysis. The toluene apparently does not interfere with the activity of the enzyme.

The cytochrome peroxidase is precipitated from the supernatant of the autolysate by adding ammonium sulfate and trichloroacetic acid. The resulting precipitate is extracted with water and dialyzed. Upon centrifugation of the dialysate, a clear solution containing the enzyme is obtained.

The next step in the purification involves fractionation with ethyl alcohol. It was found that best results were obtained only when the solution was as salt-free as possible. A dry powder which is stable for months, when kept in a vacuum desiccator at 0°, is obtained.

All succeeding purifications are obtained by fractional adsorption on γ-aluminum hydroxide (2). A solution of the alcohol-dry powder is adsorbed on the alumina and eluted with dilute ammonium sulfate. The eluate is dialyzed until salt-free and the pro-

cedure repeated with somewhat different conditions of adsorption and elution. The final eluate is dialyzed and is stable for weeks if kept at 0°.

Procedure

Extraction—Anheuser-Busch's bakers' yeast is washed by filtration and pressed dry. This yeast is dried at room temperature and ground in a coffee-mill. 1 kilo of yeast, 3 liters of water, and 75 cc. of toluene are mixed and allowed to autolyze at 25° for 24 hours, and then placed in a cold room overnight. This mixture is centrifuged, yielding a cloudy supernatant liquid (Solution A) containing a total of 400,000 units of cytochrome peroxidase activity with a Q of 2 to 4.

Ammonium Sulfate-Trichloroacetic Acid Precipitation—350 gm. of ammonium sulfate and 55 cc. of 20 per cent trichloroacetic acid solution are added to every liter of Solution A at 0°. All the following operations are carried out at this temperature. The precipitate is extracted with water and the extract dialyzed in the cold for 15 hours. The cloudy solution is centrifuged, yielding Solution B which has Q 20 to 30; yield about 45 per cent.

Alcohol Fractionation—Solution B is diluted to a concentration of 5 mg. of protein per cc., and the pH adjusted to 4.2. Ethyl alcohol is added until the concentration is 19 per cent. The resulting precipitate is discarded and the alcohol content increased to 38 per cent. The precipitate is collected and dried *in vacuo*. 20 mg. of this dry powder are suspended in 1 cc. of water and centrifuged. The resulting supernatant (Solution C) has a Q of 80 to 100; yield about 30 per cent.

First Adsorption and Elution—Solution C is adsorbed upon γ-aluminum hydroxide (1 cc. of hydroxide suspension of dry weight 7.6 mg. per cc. is used for 5 cc. of Solution C). The enzyme is eluted with successive small amounts of 18 per cent saturated ammonium sulfate solution. The eluate is dialyzed 36 hours and then centrifuged to give clear golden yellow Solution D with a Q of 700 to 1000; yield about 30 per cent.

Second Adsorption and Elution—Solution D is adsorbed upon γ-aluminum hydroxide (1 cc. of hydroxide suspension for 20 cc. of Solution D). The enzyme is eluted this time with small quantities of 5 per cent saturated ammonium sulfate solution. Two elutions

with one-tenth the volume of the original Solution D are generally sufficient. The combined eluates are dialyzed 24 hours against running distilled water and are then centrifuged to give clear Solution E with a Q of 1700 to 2700; yield about 50 per cent.

The most active preparation thus obtained is more than 3-fold purer than the best product obtained by the previous procedure. Approximately 500- to 1000-fold purification on the basis of the original extract has been effected by these five steps.

Properties

Absorption Spectrum—The spectrum of cytochrome *c* peroxidase, as shown in Fig. 1, has three absorption peaks, an intense band in the blue at 4100 Å. and two shallow bands at 5000 and 6200 Å. The measurements were made on a preparation of $Q = 2280$. In the previously published spectrum (1) of a preparation of much lower purity, $Q = 800$, the 4100 Å. band appeared as in the purer preparation, but the weak, diffuse bands at 5000 and 6200 Å. were masked by general absorption. This three-banded spectrum is somewhat similar to those given in the literature for horseradish peroxidase preparations, but it is not identical. Keilin and Mann (3) report a peroxidase spectrum consisting of four bands at 4980, 5480, 5830, and 6450 Å. Recently Theorell (4) has succeeded in further purifying horseradish peroxidase and has separated it into two components, one having two bands at 4980 and 6400 Å., the other at 5480 and 5830 Å. None of these authors gives any information concerning absorption in the Soret region. Itoh (5) reports a peak at 4000 Å., while Kuhn, Hand, and Florkin (6) place this band at 4150 to 4200 Å. In Table I the positions of the absorption bands of the various peroxidases are summarized. The spectrum of cytochrome *c* peroxidase approaches most closely that of Theorell's Component II. The band at 5000 Å. agrees very closely with the Component II band at 4980 Å. There is, however, a definite difference in the position of the bands in the red; namely, 6400 Å. for Theorell's peroxidase and 6200 Å. for cytochrome *c* peroxidase.

When sodium hydrosulfite is added to peroxidases, the ferric iron is reduced to the ferrous state. Keilin and Mann (3) report that reduced horseradish peroxidase has two bands in the visible region, at 5580 and 5945 Å. It has been shown (1) that reduced

cytochrome *c* peroxidase has a Soret band at 4375 Å. and a strong band in the visible region at 5600 Å. There is no trace of a band

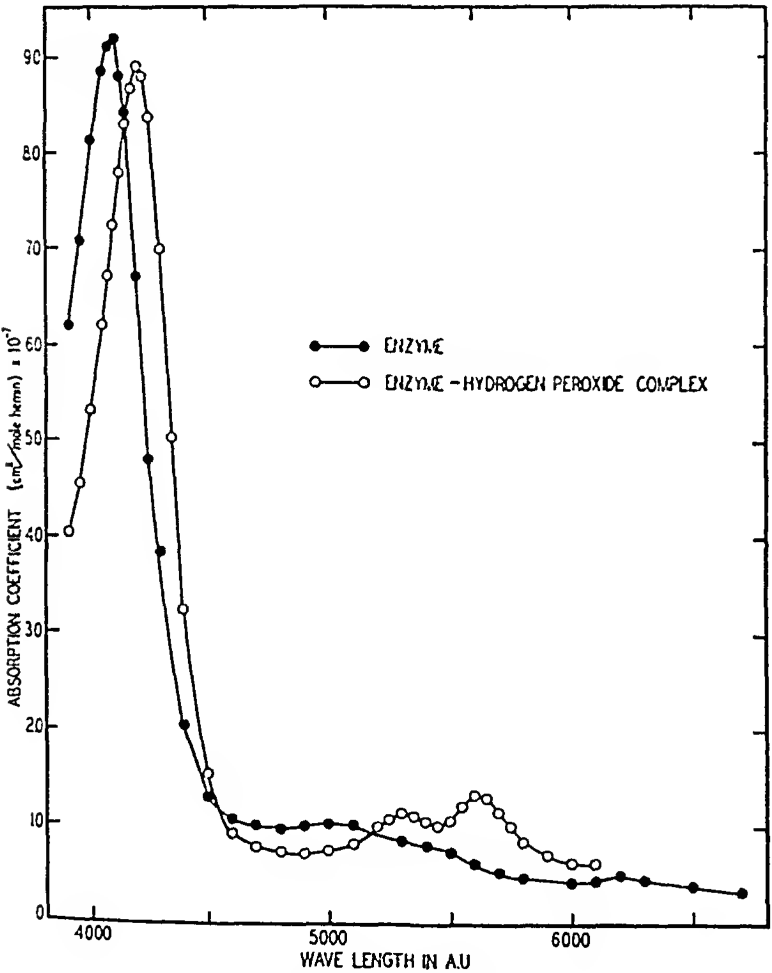

FIG. 1. Spectrum of cytochrome *c* peroxidase and of the cytochrome *c* peroxidase-hydrogen peroxide complex.

at 5945 Å. However, a change in inflection at 6700 Å. indicates that possibly a very weak band is present at that wave-length.

The foregoing comparisons demonstrate that cytochrome *c* per-

oxidase has a distinctive spectrum which is not identical with that of any other published peroxidase spectrum.

Absorption and Activity—Because of the greater purification achieved, it is now possible to demonstrate that the activity of the purified enzyme is directly related to its absorption in the Soret

TABLE I

Spectra of Various Peroxidases in Oxidized Form, in Reduced Form, and in Hydrogen Peroxide Complex

Compound	Absorption bands	Bibliographic reference
	Å.	
Cytochrome *c* peroxidase	4100, 5000, 6200	
Horseradish "	4980, 5480, 5830, 6450	Keilin (3)
Peroxidase I	5480, 5830	Theorell (4)
" II	4980, 6400	" (4)
Horseradish peroxidase	4000	Itoh (5)
" "	4150–4200	Kuhn, Hand, Florkin (6)
Reduced cytochrome *c* peroxidase	4375, 5600	Altschul, Abrams, Hogness (1)
Reduced horseradish peroxidase	5580, 5945	Keilin (3)
Cytochrome *c* peroxidase-H_2O_2 complex	4200, 5300, 5600	
Horseradish peroxidase-H_2O_2 Complex I*	5305, 5610	Keilin (3)
Horseradish peroxidase-H_2O_2 Complex II*	5455, 5830	" (3)

* Complex I here designates the compound formed by the addition of an equimolecular amount of hydrogen peroxide to peroxidase. Complex II is the compound existing in the presence of a large excess of hydrogen peroxide.

region. This is shown in Table II which represents results obtained from a number of preparations. It can readily be seen that for preparations with values of Q exceeding 800 the relation of absorption to activity is constant. This indicates that other iron prophyrin compounds can be present only in very small amounts.

Hemin Content—As is shown in Table III, enzyme activity is directly proportional to hemin content. We can therefore con-

clude that cytochrome *c* peroxidase is a hemin-protein compound. It is interesting to note that Theorell has been able to split reversibly the hemin prosthetic group from his Component II peroxidase preparation.

Copper Content—In the previous paper, it was shown that the

TABLE II

Relation between Enzyme Activity and Light Absorption

Q	Activity per cc.	$\log \left(\frac{I_0}{I}\right)^*_{410}$	$\frac{\log I_0/I}{\text{Activity per cc.}}$
			$\times 10^{-4}$
445	910	0.456	5.01
710	1040	0.533	5.13
843	2100	0.891	4.25
1035	1170	0.488	4.17
1320	1610	0.662	4.12
2230	3380	1.395	4.13

* 1 cm. cells were used for the tests. Readings were taken at 4100 Å., with a photoelectric spectrophotometer.

TABLE III

Relation between Enzyme Activity and Hemin Content

The hemin content was determined spectrophotometrically by conversion to reduced pyridine hemochromogen (1).

Q	Per cent hemin	$\frac{\text{Per cent hemin}}{Q}$
		$\times 10^{-4}$
445	0.14	3.1
705	0.27	3.8
806	0.27	3.3
843	0.26	3.1
1035	0.30	2.9
2230	0.75	3.2

peroxidase preparations contained small amounts of copper; the activity, however, was not impaired by huge excesses of sodium diethyl dithiocarbamate, a specific copper inhibitor. We have been unable to show any relationship between enzyme activity and copper content. Thus two preparations of very different degrees of purity (Q = 445 and 1035) had the same copper content (0.25

per cent). Another preparation of $Q = 806$ had a copper content of 0.09 per cent. It is quite possible that the copper accumulates during the long periods of dialysis.

Enzyme-Substrate Complex

Cytochrome *c* peroxidase forms a stable complex with hydrogen peroxide. Addition of hydrogen peroxide causes the brown cytochrome *c* peroxidase solution to turn distinctly red. The changes in spectrum were determined by use of a photoelectric spectrophotometer, with an enzyme preparation with Q 2230 units per mg., a hemin content of 0.75 per cent, an activity of 3380 units per cc., and a protein content of 1.37 mg. per cc.

0.5 cc. of the enzyme solution was placed in a small absorption cell (capacity 0.6 cc.) 1 cm. in length. The spectrum was taken over the visible range at a temperature of 5°. This low temperature prevented denaturation of the enzyme solution. After the entire spectrum was obtained, 0.1 cc. of 0.1 M hydrogen peroxide was added to the 0.5 cc. of enzyme solution in the absorption cell and the spectrum again observed.

Both spectra are plotted in Fig. 1. The spectrum for the H_2O_2 complex has been corrected for the dilution produced by the addition of hydrogen peroxide. Clearly, the addition of hydrogen peroxide has resulted in the formation of a compound with a different spectrum, as is shown in Table I.

Keilin and Mann have investigated complex formation between horseradish peroxidase and hydrogen peroxide. As is indicated in Table I, they found two spectroscopically distinct compounds. The first, designated in Table I as Complex I is formed when 1 mole of hydrogen peroxide is added per mole of peroxidase hemin. However, when a large excess of peroxide is added, the spectrum shifts to that of the one designated as Complex II. The spectrum of the cytochrome peroxidase-H_2O_2 complex is almost identical with that of Complex I. This spectrum is obtained in the presence of a large excess of hydrogen peroxide and there is no evidence for a second spectrum corresponding to that of Keilin and Mann's Complex II.

In the above spectroscopic determination a large excess of hydrogen peroxide was used. In order to determine how many molecules of hydrogen peroxide were taken up per molecule of hemin

in the enzyme preparation, the enzyme was titrated with small amounts of H_2O_2. 0.5 cc. of enzyme was placed in a 1 cm. absorption cell and the absorption measured spectrophotometrically at 5600 Å. Small amounts of hydrogen peroxide were added until the absorption reached a maximum, indicating that all the enzyme was in the form of the complex. The results of the titration are shown in Fig. 2.

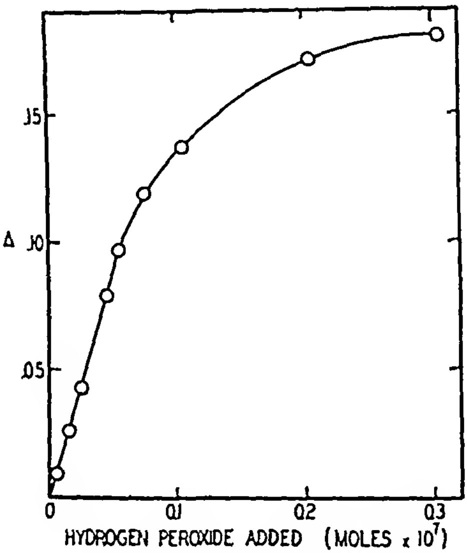

FIG. 2. Titration of cytochrome c peroxidase with hydrogen peroxide. The ordinate, Δ, is the difference between log I_0/I for the enzyme-hydrogen peroxide mixture and log I_0/I for the enzyme alone before any hydrogen peroxide had been added.

From the experimental data thus obtained, the following properties of cytochrome peroxidase can be determined, (a) the number of moles of hydrogen peroxide which combine with 1 mole of enzyme, (b) the dissociation constant of the enzyme-H_2O_2 complex, (c) the maximum molecular weight of cytochrome peroxidase (assuming one heme group per molecule). For the simplest case, *i.e.* 1 mole of enzyme reacting with 1 mole of H_2O_2, the dissociation constant, K_D, is expressed in terms of concentrations of enzyme, substrate, and complex, as follows:

$$e + s = es \tag{1}$$

where e represents the total enzyme, s the total substrate (H_2O_2), and es the complex.

$$K_D = \frac{(e - es)(s - es)}{(es)} \tag{2}$$

$$K_D = \left[\frac{(e)(s)}{(es)} + (es)\right] - (s) - (e) \tag{3}$$

It is possible, however, from a consideration of Beer's law, to transform Equation 3 so that K_D is expressed in terms of the experimentally determined quantities shown in Fig. 2.

When no hydrogen peroxide is added, the observed absorption is related to the enzyme concentration as follows:

$$\text{Log}\left(\frac{I_0}{I}\right) = \alpha_e(e) \tag{4}$$

where log I_0/I is the observed absorption, α_e is the absorption coefficient of cytochrome peroxidase at 5600 Å., and (e) is the concentration of enzyme. In a mixture of hydrogen peroxide and the enzyme, the observed absorption is the sum of the absorption due to the free enzyme and that of the complex.

$$\text{Log}\left(\frac{I_0}{I}\right)_m = \alpha_e[(e) - (es)] + \alpha_{es}(es) \tag{5}$$

where log $(I_0/I)_m$ is the absorption of the mixture, (es) is the concentration of the complex, and α_{es} is the absorption coefficient of the complex. The quantity Δ is defined as follows:

$$\Delta = \log\left(\frac{I_0}{I}\right)_m - \log\left(\frac{I_0}{I}\right) = (\alpha_{es} - \alpha_e)(es) = k(es) \tag{6}$$

Therefore

$$(es) = \frac{\Delta}{k}$$

When all the enzyme is in form of the complex, the concentration of enzyme is given by

$$(e) = (es) = \frac{\Delta_{max}}{k} \tag{7}$$

where Δ_{max} is the greatest change in absorption observed and corresponds to the horizontal flat portion in Fig. 2. By substituting the

values of (es) and (e) from Equations 6 and 7 in Equation 3, we obtain,

$$K_D + (s) + \frac{\Delta_{\max}}{k} = \left[\frac{\frac{\Delta_{\max}}{k}(s)}{\frac{\Delta}{k}} + \frac{\Delta}{k}\right] \tag{8}$$

or

$$K_D + \left(s - \frac{\Delta_{\max}}{\Delta}(s)\right) = \frac{1}{k}(\Delta - \Delta_{\max}) \tag{9}$$

or

$$\left(\frac{\Delta_{\max}}{\Delta} - 1\right)(s) = \frac{1}{k}(\Delta_{\max} - \Delta) + K_D \tag{10}$$

If, therefore, $(\Delta_{\max}/\Delta - 1)(s)$ is plotted against $(\Delta_{\max} - \Delta)$, a straight line should be obtained, the ordinate intercept of which is equal to $(+K_D)$ and the slope is equal to $1/k$.

In Table IV are given the values calculated from the data in Fig. 2 necessary to determine K_D and $1/k$, and in Fig. 3, $(\Delta_{\max}/\Delta - 1)(s)$ is plotted against $(\Delta_{\max} - \Delta)$. Obviously the points lie on a straight line; the value of $K_D = 1 \times 10^{-6}$ mole per liter and $k = 9.6 \times 10^6$ cm.^{2} per mole. We can therefore conclude that the assumption in Equation 1 is correct and that 1 mole of enzyme combines with 1 mole of hydrogen peroxide.

The concentration of the enzyme calculated from the value of k is 1.9×10^{-8} mole of enzyme per cc. For the same enzyme solution, the hemin content was determined to be 1.5×10^{-8} mole per cc. We can therefore conclude that within the limits of experimental error each mole of enzyme contains 1 mole of hemin.

The maximum molecular weight of the cytochrome c peroxidase is calculated from the fact that 1.9×10^{-8} mole of enzyme is equivalent to 1.4×10^{-3} gm. of protein. As a result the maximum molecular weight is determined to be 71,000. The purest preparation ever obtained had a Q of 2700 and on the above basis would have a maximum molecular weight of about 60,000.

The maximum molecular weight when compared with other hemin enzymes throws some light on the purity of the peroxidase. Pure catalase, for example, has a value of about 62,000 per atom of

hemin iron. Peroxidase Component II of Theorell is somewhat lower; namely, 48,000. A group of hemin-proteins, including

TABLE IV

Calculation of Dissociation Constant of Enzyme-Substrate Complex

$\frac{\Delta_{max}}{\Delta}$	$\frac{\Delta_{max}}{\Delta} - 1$	S*	$\left(\frac{\Delta_{max}}{\Delta} - 1\right)S$	$\Delta_{max} - \Delta$
		$\times 10^{-5}$	$\times 10^{-5}$	
20.6	19.6	0.09	1.76	0.176
7.1	6.1	0.29	1.77	0.159
4.3	3.3	0.48	1.58	0.142
2.3	1.3	0.83	1.08	0.106
1.9	0.9	0.99	0.89	0.088
1.56	0.56	1.30	0.73	0.066
1.35	0.35	1.74	0.61	0.048
1.08	0.08	2.91	0.23	0.014
1.03	0.03	3.79	0.114	0.005

* The substrate concentration, S, is expressed in moles per liter.

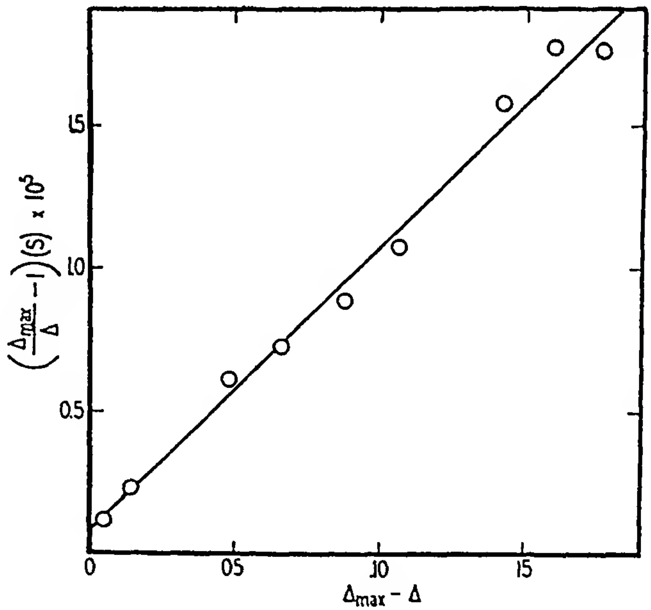

FIG. 3. Determination of the dissociation constant of the cytochrome c peroxidase-hydrogen peroxide complex.

hemoglobin, myoglobin, and cytochrome c, had much lower values of the order of magnitude of 16,000. In all probability cytochrome c peroxidase is similar to the other hydrogen peroxide-utilizing

enzymes (catalase and horseradish peroxidase), and, by the foregoing procedure, was obtained in a very nearly pure state.

Another closely related criterion of purity is the intensity of the absorption band in the near ultraviolet region. In this respect the extinction coefficients of cytochrome peroxidase are very nearly the same as those of other pure hemin-proteins and thus the peroxidase must be of a similar degree of purity.

Horseradish peroxidase has been accepted (3) as an example of a hemin enzyme in which the iron undergoes no valence change during the course of the reaction. However, it should be noted that the spectrum of the enzyme-peroxide complex is very similar to that of oxyhemoglobin and oxymyoglobin. There is no direct evidence that the iron in the complex is in the ferric state. Experiments to determine the magnetic susceptibility of this compound are in order.

If we had been dealing with a pure enzyme, k should be equal to $\alpha_{es} - \alpha_e$ (Equation 6), and if the iron in the complex were in the reduced state, and that of the uncombined enzyme in the oxidized state, then we might expect that the value of $\alpha_{es} - \alpha_e$ would be similar to the difference between that of oxymyoglobin and metmyoglobin. Our value for $\alpha_{es} - \alpha_e$ is equal to 9.6×10^6 moles^{-1} cm.^{2}, while the corresponding values for hemoglobin and for myoglobin are 10.3×10^6 moles^{-1} cm.^{2} and 9.0×10^6 moles^{-1} cm.^{2} respectively. The close agreement in the values of $\alpha_{es} - \alpha_e$ is not only indicative of the high degree of purity of the enzyme, but also provides another suggestion that perhaps the iron in the complex is reduced as in oxyhemoglobin and oxymyoglobin.

SUMMARY

1. A modified method for isolating cytochrome *c* peroxidase from bakers' yeast is reported which yields a 3-fold purer product than the original procedure.

2. The absorption spectrum of cytochrome *c* peroxidase is described as consisting of an intense band at 4100 Å. and two shallow bands at 5000 and 6200 Å.

3. Evidence is presented that cytochrome *c* peroxidase is a hemin-protein, enzyme activity being proportional to Soret absorption at 4100 Å. as well as to hemin content.

4. There is no apparent relation between copper content and enzyme activity.

5. The existence of a cytochrome c peroxidase-hydrogen peroxide complex is demonstrated and its spectrum shown to consist of three bands at 4200, 5300, and 5600 Å.

6. The dissociation constant of the enzyme-substrate complex is shown to be 1×10^{-6} moles per liter.

7. The maximum molecular weight (assuming one heme group per molecule) is shown to be 60,000. Several criteria are presented which indicate the high state of purity of the enzyme.

We wish to acknowledge our indebtedness to Anheuser-Busch, Inc., for their cooperation in supplying yeast, to the Works Progress Administration for assistance, and particularly to the Rockefeller Foundation for the grant-in-aid which made this work possible.

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ADAPTATION OF THE SCUDI COLORIMETRIC METHOD FOR PYRIDOXINE*

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A colorimetric method for the determination of pyridoxine with 2,6-dichloroquinonechloroimide has recently been described in detail by Scudi (1). This method had earlier been applied to a study of the urinary excretion of pyridoxine in the rat by Scudi and his associates (2). In an attempt to apply this general procedure to various pharmaceutical and biological materials, it developed that many substances interfere with the test. A study has been made of the modifications necessary to avoid interferences and to adapt the method to routine assays of these materials.

It was found that the most effective treatment of samples to free them from interfering substances was to adsorb the pyridoxine on superfiltrol,[1] and then elute and carry out the color reaction on the eluate. Adsorption of pyridoxine on superfiltrol proved to be most complete at approximately pH 3.0, as shown by the data in Table I. Adsorption was practically complete between pH 2.0 and 5.0, but dropped off sharply at higher pH. The data presented in Table II indicate that about 0.1 gm. of superfiltrol is required to adsorb completely 100 γ of pyridoxine. The ratio of the amount of superfiltrol used to the pyridoxine content of the sample has to be controlled within rather narrow limits, since too small an amount of the former will fail to adsorb all the pyridoxine, whereas too large an excess will produce low results owing to poor elution. If the approximate pyridoxine content of the starting sample is unknown, it is necessary to make a preliminary test, with a series of different amounts of superfiltrol to determine the optimum amount.

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[1] Obtainable from the Filtrol Corporation, 315 West Fifth Street, Los Angeles.

Various methods of eluting the pyridoxine from the superfiltrol were studied. The most satisfactory proved to be simultaneous elution and color development. This was effected by adding the butanol solution of 2,6-dichloroquinonechloroimide to the superfiltrol on which the pyridoxine had been adsorbed. Following this, veronal buffer was added, which brought the pH to 7.8 to 8.0 and

Table I

Effect of pH on Adsorption of 100 γ of Pyridoxine on 0.04 Gm. of Superfiltrol

Test No.	pH	Extinction of eluate, λ 623 mμ	Maximum
			per cent
1	2.0	1.17	95.4
2	3.4	1.24	100
3	4.8	1.17	95.4
4	6.2	0.49	39.5

Table II

Amount of Superfiltrol Required for Adsorption of 100 γ of Pyridoxine at pH 4.4

Test No.	Superfiltrol	Color due to unadsorbed pyridoxine	Adsorption
	gm.		
1	0.05	Slight blue	Incomplete
2	0.06	" "	"
3	0.07	Very slight blue	"
3	0.08	" " "	"
5	0.09	No blue	Complete
6	0.10	" "	"
7	0.11	" "	"
8	0.12	" "	"

allowed the characteristic blue color to develop. The method is as follows:

Graded amounts of unknown, estimated to contain 5 to 100 γ of pyridoxine are weighed or measured into 16 mm. calibrated test-tubes. Graded amounts of diluted pure pyridoxine are measured into a similar set of tubes to serve as standards. 2 cc. of McIlvaine's citrate-phosphate buffer (pH 3.0) are added to each tube and the volume made up to 5 cc. with water. 1 mg. of superfiltrol

is added for each microgram of estimated pyridoxine. The tubes are stoppered and shaken vigorously several times during a 30 minute period, after which the suspension is centrifuged and the liquid decanted. The adsorbates are now washed with 5 cc. amounts of solution buffered as before, by shaking, centrifuging, and decanting.

10 cc. of a butanol solution containing 62.5 γ of 2,6-dichloroquinonechloroimide per cc. are added to the tubes containing the adsorbates. After 5 minutes, during which the tubes are shaken vigorously several times, 3 cc. of a veronal buffer solution (pH 7.8) are added to each and the mixture agitated. The buffer solution is added to succeeding tubes at regular time intervals of 30 to 60 seconds, since the development of color before reading must be carefully timed. The resulting butanol-water emulsion is separated by centrifuging. The butanol layer is decanted into a colorimeter tube and the amount of color determined in an electric photometer with a filter transmitting at approximately 650 mμ. The maximum color occurs about 20 minutes after the dye solution is added. The readings are made in the same order in which the veronal buffer was added to the tubes, and at the same time intervals. Thus the color in all the tubes develops for the same length of time.

From the photometer readings obtained for the tubes containing known amounts of pyridoxine a standard curve is drawn. The pyridoxine content of each unknown tube is determined in terms of the standard by reference to the curve. An average of three to four closely agreeing values obtained in this manner is taken for calculating the pyridoxine content of the sample.

On some samples of natural products, especially those which are dark colored and low in pyridoxine content, a preliminary treatment is necessary before the above assay method can be carried out. The procedure followed in cases of this kind has been to dissolve or suspend 1 gm. of the finely powdered material in about 8 cc. of water in a test-tube, add 1 cc. of 10 per cent lead acetate, dilute to 10 cc., mix, and centrifuge. An aliquot of the supernatant solution is taken for the test as outlined.

A study was made of the performance of the assay procedure when carried out on pure solutions of pyridoxine to which other vitamins had been added. The data from these experiments are

presented in Table III. The presence of 50 or 500 γ of thiamine, riboflavin, nicotinic acid, sodium pantothenate, or 10 times these

TABLE III

Recovery of Pyridoxine in Presence of Added Vitamins

Test No.	Vitamin added per tube*	Extinction, λ 623 mμ	Recovery
			per cent
1	Control	0.50	
2	"	0.50	
3	50 γ thiamine	0.48	96
4	500 " "	0.49	98
5	50 " riboflavin	0.48	96
6	500 " "	0.51	102
7	50 " nicotinic acid	0.48	96
8	500 " " "	0.46	92
9	50 " sodium pantothenate	0.49	98
10	500 " " "	0.49	98
11	500 " ascorbic acid	0.50	100
12	5000 " " "	0.52	104

* Each tube contained 50 γ of pyridoxine.

TABLE IV

Recovery of Pyridoxine in Presence of Combined Vitamins

Test No.	Vitamins added*					Extinction, λ 623 mμ	Recovery
	Sodium panto-thenate	Thiamine	Riboflavin	Nicotinic acid	Ascorbic acid		
	γ	γ	γ	γ	γ		*per cent*
1						0.63	
2	100					0.62	99
3	100	100				0.62	99
4	100	100	100			0.63	100
5	100	100	100	100		0.60	95
6	100	100	100	500		0.60	95
7	100	100	100	500	500	0.59	94
8	100	100	100	500	2500	0.61	97

* Each tube contained 50 γ of pyridoxine.

amounts of ascorbic acid did not influence the accuracy of the assay. It should be noted here that it is impossible to carry out the assay on materials containing ascorbic acid without first elimi-

nating it by the adsorption and elution technique, or some other suitable procedure, since it destroys the blue color produced in the test.

TABLE V

Stability of Pyridoxine. Effect of Autoclaving at Different pH

Test No.	pH of solution*	Time of heating	Extinction, λ 623 mμ	Recovery
		min.		*per cent*
A1	4.6	0	0.53	100
2	4.6	5	0.50	94
3	4.6	15	0.50	94
4	4.6	45	0.53	100
B1	7.6	0	0.52	100
2	7.6	5	0.49	94
3	7.6	15	0.49	94
4	7.6	45	0.48	92
C1	9.7	0	0.49	100
2	9.7	5	0.48	98
3	9.7	15	0.49	100
4	9.7	45	0.45	92

* All solutions contained 20 γ of pyridoxine per cc.

TABLE VI

Results of Typical Pyridoxine Determinations

Sample	Pyridoxine found
	γ per gm.
Rice polish concentrate, desiccated (Labco)	71
Yeast extract, desiccated (Difco)	40
Rice bran extract (Vitab)	124
Wheat germ extract	49
	γ per cc.
Urine, before ingestion of vitamin B_6	<1
Same 1 hr. after ingestion of 100 mg. vitamin B_6	19.1

In a further study the vitamins mentioned above were combined in different ways with pure pyridoxine and recovery assays carried out. The results, shown in Table IV, indicate that no combination of these pure vitamins with pyridoxine interferes significantly with the determination of the latter by this method.

Pyridoxine has been stated to be unstable in dilute solutions to autoclaving at neutral pH. Data obtained (Table V) by the method described do not indicate that it is very unstable to heat, even in fairly alkaline solution. Solutions containing 20 γ of pyridoxine per cc. did not drop significantly in potency even after being autoclaved 45 minutes at pH 9.7.

In Table VI are presented results from a few typical determinations of pyridoxine. The method, being rapid and requiring no complicated manipulations, has proved to be particularly valuable for routine assay of natural materials as well as pharmaceutical products whose vitamin B_6 content has been augmented by the addition of crystalline pyridoxine.

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THE METABOLISM OF VITAMIN B_6

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In previous experiments it was shown that the rat eliminates 50 to 70 per cent of varied test doses of pyridoxine by way of the urinary tract (1). In the dog 20 per cent of the vitamin was recovered within 6 hours after the oral or intravenous administration of varied doses, while only 8.7 per cent of a 50 mg. intravenous dose was recovered from the urine of apparently healthy, male subjects (2). These experiments performed by means of the indophenol reaction of vitamin B_6 (3) were concerned only with the excretion of unchanged pyridoxine. We report here evidence for the metabolism of pyridoxine in man, the dog, and the rat.

EXPERIMENTAL

Four fasting, male, mongrel dogs, weighing 11.0 to 14.3 kilos, were given 200 cc. of water by stomach tube and the bladder was emptied by catheter. After 1 hour, control samples of urine were taken by catheter and each dog was given 500 mg. of pyridoxine in 250 cc. of water by stomach tube. Postadministrative samples of urine were collected over a 4 or 24 hour period, the bladders being emptied finally by catheter.

Urine samples were adjusted to pH 6.8 to 7.2 and diluted to give vitamin concentrations of approximately 5 γ per cc. The samples were then analyzed by use of a veronal buffer as previously reported (1, 2). At urine dilutions of 1:10 and above, urinary phenols do not interfere, as indicated by the negative results obtained with control samples of urine. The values obtained with the postadministrative samples are shown in Column 2 of Table I. These values represent the free "vitamin B_6" output.

That these values represent chiefly vitamin B_6 was confirmed

by the following isolation experiment. 2 gm. of the vitamin were administered to a fifth dog and a sample of urine (160 cc.) was ob-

TABLE I

Urinary Output of Vitamin B_6

Subject	Sample	Volume	Free vitamin B_6		Total vitamin B_6	
			Metabolite (1)	Vitamin B_6 (2)	Metabolite (3)	Vitamin B_6 (4)
		cc.	*mg.*	*mg.*	*mg.*	*mg.*
Dog I	Control	35	0.0	0.0	0.0	0.0
	4 hrs. Pa.*	66	3.0	29.7	8.4	172.0
" II	Control	12	0.0	0.0	0.0	0.0
	4 hrs. Pa.	138	3.1	62.2	6.9	173.0
" III	Control	39	0.0	0.0	0.0	0.0
	4 hrs. Pa.	163	5.9	113.0	7.1	144.0
" IV	Control	128	0.0	0.0	0.0	0.0
	24 hrs. Pa.	238	7.1	113.0	10.0	140.0
Human I	Control	196	0.0	0.0	0.0	0.0
	65 min. Pa.	167	1.6	51.3	2.5	72.0
	135 " "	68	2.6	54.7	4.5	93.5
" II	Control	60	0.0	0.0		
	90 min. Pa.	70	4.2	51.0		
" III	Control	96	0.0	0.0	0.0	0.0
	2 hrs. Pa.	328	3.7	95.0	4.7	149.0
" IV	Control	86	0.0	0.0	0.0	0.0
	2 hrs. Pa.	138	3.4	128.0	5.2	198.0
" V	Control	55	0.0	0.0	0.0	0.0
	3 hrs. Pa.	360	7.6	129.0	12.2	182.0
" VI	Control	130	0.0	0.0	0.0	0.0
	3 hrs. Pa.	259	7.1	132.0	11.1	185.0
Rats I	Control	15.0	0.0	0.0	0.0	0.0
	7 hrs. Pa.	28.5	0.57	21.6	0.50	23.9
" II	Control	14.5	0.0	0.0	0.0	0.0
	7 hrs. Pa.	27.5	0.50	23.1	0.48	24.2
" III	Control	20.0	0.0	0.0	0.0	0.0
	7 hrs. Pa.	25.0	0.50	24.0	0.59	25.0
" IV	Control	14.0	0.0	0.0	0.0	0.0
	7 hrs. Pa.	25.0	0.40	21.0	0.34	22.0

* Pa. = postadministrative.

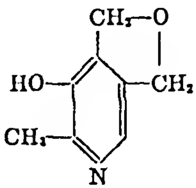
tained which showed approximately 500 mg. of vitamin B_6 as measured by the indophenol reaction. The urine was extracted continuously for 12 hours with butanol, and the extract was con-

centrated to dryness *in vacuo*. The residue was extracted with ether, and the vitamin was precipitated as the hydrochloride by passing hydrogen chloride through the dried ethereal solution. Approximately 150 mg. of material was obtained. The product on recrystallization from absolute alcohol melted with decomposition at 206–208°, corrected, and at the same temperatures when mixed with a pure sample of the vitamin.

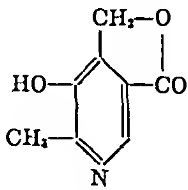
Analysis—$C_8H_{11}NO_3 \cdot HCl$.	Calculated.	C 46.7, H 5.88, N 6.81
	Found.	" 46.43, " 5.86, " 6.63

The output of vitamin B_6 as measured by the indophenol reaction carried out with a veronal buffer can include certain metabolites of the vitamin. An investigation of these metabolites was therefore undertaken.

It has been shown that vitamin B_6 does not give the indophenol reaction in the presence of a borate buffer because the phenolic group of the vitamin is masked by the formation of a complex involving boron linked to 2 molecules of the vitamin through the oxygen atoms in the 3 and 4 positions (4). The urine samples (diluted to contain approximately 50 γ of the vitamin) were therefore analyzed with a borate buffer.[1] Standard solutions of the inner ether (I) were used for the comparison, as previously described (3). Analysis showed a vitamin B_6 metabolite which,



(I)



(II)

like the inner ether, gave the indophenol reaction in the presence of the borate buffer. The values obtained are shown in Column 1 of Table I. This metabolite is measured with the free vitamin

[1] This borate buffer was prepared as follows: 50 cc. of a solution which is 1.2 M in boric acid and 1.2 M in KCl is diluted, and 2.5 N NaOH is added to pH 8.6 to thymol blue. The volume is then adjusted to 200 cc. This buffer gives a negative indophenol reaction with vitamin B_6 solutions containing 200 γ per cc. or less.

when a veronal buffer is used. Thus, subtracting the value obtained when a borate buffer is used from the value obtained with a veronal buffer gives the true value of free vitamin B_6.

Certain inferences can be drawn concerning the structure of this metabolite. The ring nitrogen of the vitamin is not substituted, and it is probable that the 2-methyl group is not altered. The 3-hydroxyl group is intact, whereas the 4-hydroxymethyl group is certainly altered. The 5-hydroxymethyl group may or may not be altered and the 6 position is unchanged. The metabolite, like vitamin B_6, gives an indophenol reaction product which is rapidly and characteristically destroyed by the addition of strong alkali. The indophenol formed by the metabolite is, however, more stable than the vitamin B_6 indophenol. These requirements are met, among other possibilities, by ethers involving the 4-hydroxymethyl group, such as the inner ether (I). The lactone (II), kindly furnished by Dr. S. A. Harris, does not give an indophenol reaction in the presence of the borate buffer.

The urine samples were also analyzed after acid hydrolysis. The hydrolysis was performed as follows: 2 cc. samples of undiluted urine mixed with 2 cc. of 6 N HCl were heated in a boiling water bath for 60 minutes. This treatment does not convert vitamin B_6 to its inner ether. After the hydrolysis, the solutions were cooled, neutralized, diluted, and tested both with the veronal and with the borate buffer. The values obtained (Column 4, Table I) when the veronal buffer was used represent the total "vitamin B_6" output. These values were always higher than the free vitamin output, and the difference between the two values represents the amount of the vitamin conjugated. Since conjugation of the 3-hydroxyl group of the vitamin inhibits the indophenol reaction, it may be inferred that the vitamin is conjugated either as a glucuronide or as an ethereal sulfate, and this conjugation involves the 3-hydroxyl group of the vitamin. The values obtained when the borate buffer was used (Column 3, Table I) also showed an increase after the hydrolysis. Thus the metabolite, like the unchanged vitamin B_6, is also conjugated in part through the 3-hydroxyl group. The amount of the metabolite conjugated is determined by subtracting the free from the corresponding total values.

Similar experiments were performed with fasting, human,

male subjects. Morning specimens were discarded and 300 cc. of water were administered. A control sample of urine was collected after 60 minutes. A solution of 500 mg. of pyridoxine in 250 cc. of water was given and postadministrative samples were collected. Analyses were performed as described. The data, shown in Table I, indicate that man and the dog eliminate the same urinary excretion products.

Similar experiments were also performed with twelve rats. The animals, weighing 175 to 225 gm., were divided into four groups of three. Each rat was given 7.5 cc. of water by stomach tube, and after 3 hours control samples of urine were collected. 10 mg. of the vitamin hydrochloride in 7.5 cc. of water were then administered to each rat, and postadministrative samples of urine were collected over a 7 hour period. The data obtained (Table I) are in agreement with previous findings (1). The differences between the total and free vitamin output are within the experimental error. Consequently it appears that at this dose level the rat, unlike man and the dog, does not conjugate the vitamin. This finding is in line with the higher excretion of the free vitamin in the rat than was found in man and the dog. At the dose level studied the rat appears to convert a smaller percentage of the ingested vitamin to the metabolite, and does not conjugate this metabolite, as is the case with man and the dog.

It has been reported (3) that the 4,5-dicarboxylic analogue of the vitamin does not give the indophenol reaction because of its solubility in the alkaline, aqueous phase used in the two phase test procedure. This, and other alkali-soluble products which might be present in urine would not be measured by the indophenol reaction. If such products exist in the urine samples, and the 3-hydroxyl and 6 position remain free, it should be possible to measure these products by coupling with diazonium salts. Swaminathan's coupling procedure (5) was therefore used to analyze the samples of human urine. The total vitamin B_6 output as measured by this method did not exceed the output found by the indophenol procedure. Consequently, it would appear that there is little or none of this type of acidic metabolite in human urine.

In analogy with other pyridine compounds N-methylation of the vitamin may be anticipated. This N-methyl derivative does not give the indophenol reaction (3), and, in agreement with the

zwitter ion nature of the vitamin (6), it was observed that the N-methyl derivative does not couple with diazonium salts. It does not form cyanines (6), nor does it react with cyanogen bromide. This derivative does react with ferric chloride to give a red-brown color. However, this color reaction is not suited to quantitative use. Consequently, we are unable, at this time, to rule out the possible occurrence of N-substituted metabolites in these samples of urine.

SUMMARY

Evidence has been presented to show that both man and the dog excrete large amounts of ingested vitamin B_6 in the urine in the form of a conjugate, whereas the rat does not. This conjugate involves the 3-hydroxyl group of the vitamin and is probably a glucuronide or an ethereal sulfate. Concentrations of this material can be quantitatively measured by the indophenol reaction following hydrolysis.

A second excretion product has also been detected in the urine of man and the dog, and to a lesser extent, in rat urine. This product is conjugated by man and the dog, but not by the rat. The structure of this metabolite has been considered and it has been shown that the 4-hydroxymethyl group of the vitamin is altered to produce this compound. The metabolite can be quantitatively measured by the indophenol reaction carried out with a borate buffer. The excretion of unchanged vitamin B_6 in the dog has been confirmed by isolation. The possible occurrence of other urinary excretion products has been considered.

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QUANTITATIVE INVESTIGATIONS OF AMINO ACIDS AND PEPTIDES

VIII. THE SOLUBILITY AND SPECIFIC ROTATIONS OF *l*(−)-LEUCINE AT TWENTY-FIVE DEGREES*

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The physical data recorded in the literature for some of the naturally occurring amino acids may be grossly inaccurate because of the uncertain purity of the amino acids used in measuring physical properties. Nearly all amino acid preparations used for such purposes have been subjected to purification procedures but, as is now recognized, the methods employed in some cases were ineffective. Because of particular difficulties in the purification of natural leucine, preparations of this amino acid were notably impure. The leucine first isolated from proteins by Proust (1) and Braconnot (2), as well as the preparations made by later investigators, was purified usually by recrystallization from water or aqueous ethanol until the specific rotation, or some other physical property, was constant and the percentages of carbon, hydrogen, and nitrogen were in close agreement with the theoretical values. It is known, however, that natural leucine is not freed from certain amino acid impurities by this treatment and that its purity cannot be established, unequivocally, by means of these criteria.

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For the preceding paper in this series see the *Journal of Physical Chemistry*, in press.

The presence of a sulfur-containing impurity in natural leucine was observed by Fischer (3) who destroyed this contaminant by heating the leucine with lead oxide for 7 hours at 165°. This method is unsatisfactory for the purification of natural leucine, since the latter is racemized in the process. Although this contaminant (methionine) was isolated in pure form by Mueller (4) in 1923 and its constitution established 5 years later by Barger and Coyne (5), prior to 1936 it was not generally recognized that purified natural leucine contained appreciable quantities of methionine. The gravity of the situation in 1935 was revealed by the report of Mueller (6) that samples of natural leucine obtained from three manufacturers contained from 2.7 to 9.2 per cent of methionine and that two especially purified leucine preparations contained readily measurable amounts of sulfur.

The inadequacy of simple crystallization procedures was shown clearly by the experiments of Cohn *et al.* (7), while, according to Fox (8), attempts to purify natural leucine by esterification and fractional distillation, electrodialysis, precipitation of methionine as a mercury complex, and crystallization from acidified butyl alcohol were unsuccessful.

Two methods for the preparation of leucine, free from methionine, have been described. Fox (8) prepared leucine, which was stated to be sulfur-free and ash-free, in 50 per cent yield from formylated crude natural leucine which had been crystallized six times from water. However, supporting analytical data were not given. Natural leucine, which was stated to be free from sulfur, was prepared by Bergmann and Stein (9) from crude natural leucine nasylate (naphthalene-β-sulfonate) which had been crystallized once from 1 N hydrochloric acid and three times from water. The purified product contained 97 per cent $l(-)$-leucine, according to analysis by the solubility method; its specific rotation was +15.33° with sodium light and a 4.8 per cent solution of the amino acid in 21 per cent hydrochloric acid at 24°.

The presence of another amino acid contaminant, isomeric with leucine, in leucine preparations obtained by the ester distillation method from the 80–85° fraction was reported by Fischer (10) in 1901. The specific rotations of these crystalline fractions in 6 N hydrochloric acid at 20° with sodium light varied from about +22° to +23°. Since the specific rotation of leucine under the

same conditions is approximately +15°, while that of isoleucine is approximately +40°, it seems probable that the impurity in Fischer's leucine was isoleucine. Isoleucine was isolated from beet sugar molasses and proteins, identified, and named by Ehrlich (11) in 1904. Although Ehrlich could not separate leucine and isoleucine by fractional crystallization of the crude mixture of amino acids, or their derivatives, he was able to extract copper isoleucinate with methyl alcohol from the mixture of copper salts.

While it seems probable that the leucine prepared either from purified formylleucine or leucine nasylate was of high purity, it is desirable that the purity of these, or any other preparations of *l*(−)-leucine, be established with determined precision and accuracy. The experiments described in the present paper were undertaken with this object.

Preparation of Purified Natural l(−)-Leucine[1]

Purification of crude natural leucine by the fractional crystallization at pH 2 of the salt, dileucine monohydrochloride,[2] was the procedure first investigated. This method was abandoned when it was ascertained that the sulfur content of succeeding crystal fractions was not appreciably less than that of the crude leucine. Fractional crystallization of leucine monohydrochloride, a method suggested by Mueller,[3] was the procedure finally adopted.[4] This method is described below.

A mixture containing 114 gm. of commercial leucine[5] and 483 ml. of 8.3 N hydrochloric acid was heated until the solid dissolved.

[1] It was found by A. W Butler in this laboratory that the specific rotation, +21 5° in 6 N HCl at 25 6°, of a sample of *l*(−)-leucine isolated from casein was unchanged after two recrystallizations from hydrochloric acid. It would appear, therefore, that crude natural leucine isolated from casein is more impure and is purified less readily than that obtained from zein.

[2] The properties of *l*(−)-dileucine monohydrochloride have been investigated by Takahashi and Yaginuma (12), Barnett (13), and Hill and Robson (14).

[3] Mueller, J. H., private communication, June, 1935.

[4] The purification of natural leucine by this method was first described by Heckel (15) and Samec (16) in 1908.

[5] Technical grade *l*(−)-leucine was purchased from the Corn Products Refining Company, Argo, Illinois. The specific rotation of this material at 25° in 6 N hydrochloric acid with sodium light was +15.4°.

The suspension of crystals, which formed after the solution had stood overnight at room temperature, was filtered. The moist crystalline *l*(−)-leucine monohydrochloride was suspended in 400 ml. of 6 N hydrochloric acid and the mixture heated until the solid dissolved. After this solution had stood overnight, the suspension of crystals was filtered. This procedure was continued until no crystallization occurred (Flask 3).

A second 114 gm. portion of the commercial leucine and 83 ml. of 12 N hydrochloric acid were mixed with the filtrate obtained initially by filtering the first suspension of crystals. This mixture was heated until the solid dissolved. The next day the suspension of crystals was filtered and suspended in the second filtrate from the first series of runs. The procedure was continued as described with the third filtrate and two additional 400 ml. volumes of 6 N hydrochloric acid to dissolve the crystals. The crystals which formed in Flask 5 were collected.

After four such series of runs the filtrate in Flask 1, which had become discolored from dissolved impurities, was discarded and Flask 6 containing 400 ml. of 6 N hydrochloric acid was included in the series. Eighteen 114 gm. lots of the commercial leucine were subjected to recrystallization in this manner. The total yield of purified *l*(−)-leucine monohydrochloride was 930 gm.

922 gm. of this product were dissolved in 7 liters of hot distilled water. 10 gm. of norit A were stirred into the hot solution, the suspension was filtered, and the filtrate was brought to pH 7 by the addition of about 400 ml. of concentrated ammonium hydroxide. After this solution had stood for 12 hours in the refrigerator, the suspension of *l*(−)-leucine crystals was filtered and washed three times, a total of 2200 ml. of methanol being used. The yield of *l*(−)-leucine, dried at 60°, was 545 gm. (75 per cent of the theoretical amount).

533 gm. of this product were dissolved in 12 liters of boiling distilled water, the solution filtered, and the filtrate evaporated until crystals began to form. After the suspension had stood overnight in the refrigerator, it was filtered. The crystals were washed, a total of 1100 ml. of methanol being used. The yield of *l*(−)-leucine, dried at 50°, was 313 gm. (59 per cent recovery).

Purity of Purified Natural l(−)-Leucine

The qualitative observations, semiquantitative measurements, and quantitative analyses utilized in determining the degree of purity of the purified leucine are described below. The leucine consisted of odorless, pure white, plate-like crystals which gave a crystal-clear aqueous solution.

Moisture—The moisture content, determined by drying approximately 0.5 gm. samples to constant weight at 77° in a partial vacuum in an Abderhalden drier in the presence of calcium chloride, was 0.00 ± 0.02 per cent.

Ash—The ash content, determined by igniting approximately 1 gm. samples, was 0.00 ± 0.01 per cent.

Inorganic Impurities—The presence of ammonia, iron (ferrous and ferric), phosphate, and heavy metals (impurities commonly found in amino acids isolated from protein sources) could not be demonstrated. If they were present, the percentage of each was definitely less than 0.004. Chloride was shown to be present but its percentage was definitely less than 0.004.

The procedures used in making these tests are described in detail because of their general application to other amino acids. The stipulated reagents[6] are added to weighed amounts of the amino acid and to aliquots of standard solutions containing known weights of c.p. chemicals. The intensities of the resulting colors or turbidities are compared visually and the amounts of impurities are estimated from these data. A tolerance of 0.004 per cent of each inorganic impurity has been adopted by the present authors as an arbitrary standard which may be applied conveniently and with satisfactory accuracy to minimum quantities of amino acids. It was demonstrated that the sensitivity of the tests for inorganic impurities is not altered measurably by the presence of an amino acid. These tests are described below.

Chloride—A 50 mg. sample of the amino acid and 0.30 ml. of a solution containing 0.0067 mg. of chloride (as sodium chloride) per ml. of distilled water are placed in separate 4 inch test-tubes. To each tube are added 4 ml. of 2 N nitric acid and 0.5 ml. of 0.1

[6] Only substances which have been shown by test to be free from the impurities to be determined should be used in the preparation of reagents.

N silver nitrate solution. The solutions in each tube are mixed and, after 1 minute, the turbidities are compared.

Ammonia—A 10 mg. sample of the amino acid and 0.10 ml. of a solution containing 0.004 mg. of ammonia (as ammonium chloride) per ml. of distilled water are placed in separate 3 inch test-tubes. To each tube are added 1 ml. of 1 N sodium hydroxide solution and 1 drop of Nessler's reagent. The solutions in each tube are mixed and the orange-red colors compared immediately.

Iron—A 67 mg. sample of the amino acid and 0.50 ml. of a solution containing 0.00536 mg. of iron (as $FeCl_2 \cdot 6H_2O$) per ml. of 2 N hydrochloric acid are placed in separate 6 inch test-tubes. To each tube are added 12 ml. of 2 N hydrochloric acid and 1 ml. of 2 N nitric acid. The solutions in each tube are heated to boiling and cooled. 5 ml. of 0.1 N potassium thiocyanate solution are added to each tube and the resulting pink colors are compared immediately.

Phosphate (Calculated As P_2O_5)—A 100 mg. sample of the amino acid and 0.60 ml. of a solution containing phosphate equivalent to 0.0067 mg. of P_2O_5 (as $Na_3PO_4 \cdot 12H_2O$) per ml. of distilled water are placed in separate 4 inch test-tubes. To each tube are added 0.6 ml. of 4 N nitric acid and 0.5 ml. of ammonium molybdate solution[7] (to which an equal volume of 6 N nitric acid has been added). The solutions in each tube are mixed and heated for 15 minutes at about 60° with the tubes inclined at an angle of 45°. The yellow precipitates on the walls of the tubes are compared.

Heavy Metals—An 84 mg. sample of the amino acid and 0.50 ml. of a solution containing 0.00336 mg. of copper (as $CuSO_4 \cdot 5H_2O$) and 0.00336 mg. of nickel (as $NiCl_2 \cdot 5H_2O$) per ml. of 4 N nitric acid are placed in separate 4 inch test-tubes. 0.5 ml. of 4 N nitric acid is added to the tube containing the amino acid. To each tube are added 2 drops of concentrated ammonium hydroxide and 1.5 ml. of distilled water saturated with hydrogen sulfide. The dark colored turbidities are compared immediately.

Methionine[8]—The leucine contained 0.10 ± 0.05 per cent of

[7] Prepared by dissolving 90 gm. of C.P. ammonium molybdate in 200 ml. of 6 N ammonium hydroxide, dissolving 240 gm. of ammonium nitrate in this solution, and diluting the final solution to 1 liter with distilled water.

[8] A method, recommended for the determination of methionine present as a contaminant in preparations of natural leucine, has been reported by

methionine according to analysis by the following procedure. A 750 mg. sample of the leucine, 35 ml. of water, and 5 ml. of Benedict's sulfur reagent (18) were placed in a 200 ml., long necked, round bottomed flask. The mixture was heated with a small flame until, after an hour, a dry residue resulted. The latter was heated intensely until all of the organic material was decomposed. 10 ml. of 1 N hydrochloric acid were added to the cooled, fused mass. The suspension was heated to boiling, cooled, and filtered. 10 ml. of 1 N barium chloride solution were added to the filtrate, the mixture was stirred, and the precipitate of barium sulfate was allowed to settle for 24 hours or was centrifuged. The reagents, as well as solutions of C.P. potassium sulfate containing sulfur equivalent to 0.01, 0.03, 0.04, and 0.05 per cent of methionine in a 750 mg. sample of leucine, were subjected simultaneously to the treatment accorded the leucine. The volumes of the barium sulfate precipitates were compared visually and the percentage of methionine in the leucine estimated from these data. Since Painter and Franke (19) have shown that only about 40 per cent of the sulfur in methionine is recovered as barium sulfate by the Benedict-Denis procedure, the value for methionine was multiplied by 2.5 to obtain the true methionine content of the leucine.[9]

That analyses of the indicated precision may be made by this procedure has been substantiated by five persons working independently in this laboratory. It was shown, also, in experiments with *dl*-leucine and *dl*-methionine that 0.016 per cent methionine is the least amount of this amino acid in 750 mg. of leucine which can be determined as barium sulfate by the visual method.

The accuracy of the authors' value for the percentage of methionine in purified natural leucine was confirmed by Callan and Toennies (20)[10] who independently analyzed the leucine.[11]

Kolb and Toennies (17). Methionine was determined with an accuracy of ±0.1 per cent in mixtures of amino acids and 0.5 per cent in tissues by iodometric determination of the hydrogen peroxide consumed in the oxidation of methionine to the sulfoxide.

[9] A factor to correct for the loss of sulfur is not required if pure *dl*-methionine, rather than potassium sulfate, is used as the standard.

[10] Toennies, G., and Callan, T. P., Lankenau Hospital Research Institute, Philadelphia, private communication, September, 1940.

[11] Amino Acid Manufactures' *l*(−)-leucine, Lot No. 4, analytically pure grade.

Equivalent Weight[12]—The equivalent weight, determined by formol titration with the glass electrode and the method described by Dunn and Loshakoff (22), was 99.7 ± 0.08 per cent of the theoretical value. Samples of the purified leucine weighing 0.3881 and 0.3882 gm. were titrated with 0.3288 N sodium hydroxide in the presence of 32 ml. of carbon dioxide-free distilled water and 18 ml. of formaldehyde (J. T. Baker Chemical Company, C.P. analyzed, 35 to 40 per cent by weight). The formaldehyde, which had been nearly neutralized with base, was titrated with standard base with the glass electrode. A correction factor for the base consumed by the formaldehyde was calculated from the titration data.

Solubility—The solubility was found to be 2.19 ± 0.01 gm. per 100 gm. of water at 25.10°. Determinations were made with 110 ml. oil sample bottles and the procedure described by Dunn, Ross, and Read (23). It was assumed that equilibrium had been attained after the leucine samples had been rotated continuously for 4 days in a thermostat at 25.10 ± 0.03°. The gm. of leucine in 9.89 gm. of solution were shown to be 0.2155, 0.2153 (0.2154, average), cold side, with 2.2 gm. of solute per 50 ml. of solution; 0.2139, 0.2142 (0.21405, average), hot side, with 2.2 gm. of solute per 50 ml. of solution; 0.2134, 0.2136 (0.2135, average), cold side, with 4.4 gm. of solute per 50 ml. of solution; 0.2131, 0.2135 (0.2133, average), hot side, with 4.4 gm. of solute per 50 ml. of solution.

Specific Rotation—A 0.01° Schmidt and Haensch polarimeter was used to determine optical rotations. A 4 dm. water-jacketed polarimeter tube, which could be filled through a side opening without disturbing the setting of the end plates, was used for all determinations. Under these conditions observed rotations reproducible to 0.003° could be determined by an experienced worker. All solutions were prepared in a thermostat at 25.0°. The specific rotation data are given below.

$[\alpha]_D^{25.0} = \frac{+1.320 \times 26.206}{0.5684 \times 4.000} = +15.21°$ (+15.20°, average of four determinations with a probable error of 0.04°) in 6.08 N hydrochloric acid.

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[12] That neutral aliphatic α-amino acids may be analyzed with high precision and accuracy by the formol method was corroborated by Toennies and Callan (21) who used a perchloric acid titration procedure.

$$[\alpha]_D^{25.0} = \frac{-0.865 \times 26.206}{0.5354 \times 4.000} = -10.58°$$ (−10.57°, average of five determinations with a probable error of 0.04°) in distilled water.

No significant differences were noted in the values obtained with ordinary distilled water heated to boiling and that boiled for 2 hours.

The specific rotations of a sample of *l*(−)-leucine purified by a different method were found to be identical, within experimental error, with the values (given above) measured under the same conditions. The purification procedure and specific rotation data are given below.

Residues weighing 100 gm. from the purification of *l*(−)-leucine by the hydrochloric acid method were converted to the nasylate by treatment with naphthalene-β-sulfonic acid by the method of Bergmann and Stein (9). The nasylate was recrystallized three times from water and decomposed with ammonium hydroxide. The specific rotation of the resulting *l*(−)leucine twice recrystallized from water is as follows:

$$[\alpha]_D^{25.0} = \frac{+1.367 \times 26.293}{0.5904 \times 4.000} = +15.22°$$ (+15.19°, average of two determinations with a probable error of 0.04°) in 6.08 N hydrochloric acid.

$$[\alpha]_D^{25.0} = \frac{-0.871 \times 26.206}{0.5395 \times 4.000} = -10.58°$$ (−10.59°, average of six determinations with a probable error of 0.06°) in distilled water.

The equivalent weight obtained by formol titration of this material, as well as that of the once recrystallized *l*(−)-leucine, was about 1 per cent higher than the theoretical amount. A satisfactory explanation of this result cannot be given. This purified *l*(−)-leucine contained less than 0.1 per cent methionine.

DISCUSSION

It would appear from the experimental findings that 0.05 per cent of moisture, ash, and inorganic constituents, 0.10 per cent of methionine, and 0.5 per cent of amino acids other than leucine are the maximum amounts of these impurities which could be present in the leucine. It is apparent, however, that the determination of amino acid impurities, other than methionine, is subject to the shortcoming that it is difficult to determine with high precision the equivalent weight of leucine, which is a relatively weak acid

even in the presence of formaldehyde. Furthermore, the presence of isoleucine would not be detected by the formol titration procedure. Because of the tendency of *l*(−)-leucine to form mixed crystals with *l*(+)-isoleucine (11) and *l*(−)-methionine, it is not impossible that appreciable quantities of these amino acids may have escaped dissolution in the solubility experiments. It may be true, therefore, that the concordance of the solubility data when small and large excesses of solute are used may not be a dependable criterion of purity of the leucine.

It seems highly probable, however, that the purity of the present sample of leucine is higher than that of other preparations of this amino acid.[13] From a consideration of (*a*) the experimental observation that the specific rotation of crude leucine in 6 N acid is

Table I

Approximate Specific Rotations of Certain Amino Acids

Amino acid	$[\alpha]_D^{25}$ 6 N HCl	$[\alpha]_D^{25}$ H_2O
	degrees	*degrees*
l(+)-Isoleucine	+40	+11
l(+)-Valine	+29	+6
l(−)-Methionine	+22	−8
l(−)-Phenylalanine	−7	−35
l(−)-Leucine	+15	−10

decreased from about +20° to +15°, and in water from about −7° to −11° by fractional crystallization of its hydrochloride, nasylate, or formyl derivative, and (*b*) the approximate specific rotations of the amino acids listed in Table I, it would seem reasonable to conclude that not more than traces of these amino acids, with which crude natural leucine is commonly contaminated, could be present in the purified leucine.

It is significant, also, that with the exceptions noted the authors'

[13] According to experiments performed by E. A. Murphy in this laboratory, natural *l*(−)-leucine purified by the procedure of Fox (8) contained less than 0.004 per cent of sulfur. If these results alone are considered, leucine purified in this manner is somewhat more pure than that prepared by the method described in this paper.

TABLE II

*Specific Rotations of l(−)-Leucine**

Solute per 100 ml. solution	Solvent	Temperature	α (observed optical rotation)	$[\alpha]_D^t$	$[\alpha]_D^{25.0}$	Bibliographic reference No.
gm.		°*C.*	*degrees*	*degrees*	*degrees*	
5.01	7.4 N HCl	16		+18.9	+19.4	24
4.88	6.7 " "	20	+2.06	+19.05	+19.34	11
5.27	6.0 " "	20	+1.96	+18.5	+18.8	25
5.012	6.0 " "	16		+17.8	+18.4	24
4	6.0 " "	15	+0.71	+17.75	+18.38	26
4.5	6.0 " "	20	+1.54	+18.00	+18.32	27
5.91	6.0 " "	20	+2.09	+17.67	+17.99	25
6.4418	2.9 " "	20.2	+2.26	+17.54	+17.84	28
	6 " "	25		+16.6	+16.6	7
4	6 " "	23		+16.5†	+16.6	30
1.31	3.0 " "	20		+16.0	+16.4	31
5.1	6.0 " "	20	+0.81	+15.9‡	+16.2	29
4.03	6.0 " "	20	+1.27	+15.77	+16.09	32, 33
7	6 " "	20		+15.7	+16.0	34
4.03	6.0 " "	20	+1.26	+15.64	+15.96	32, 33
4.622	6.3 " "	20	+1.57	+15.59§	+15.91	35
4.03	6.0 " "	20	+1.25	+15.53	+15.85	32, 33
1.0927	6 " "	20		+15.51	+15.83	36
	6 " "	25		+15.8	+15.8	7
4.00	6.0 " "	20	+0.62	+15.46	+15.78	32, 33
	6 " "	25		+15.7	+15.7	7
4	6 " "	23		+15.3	+15.4	30
4.8	6 " "	24		+15.33	+15.39	9
2.006	6.0 " "	22	+1.213	+15.1	+15.3	‖
2.169	6.08 " "	25.0	+1.320	+15.21	+15.21	This paper
4.35	6 " "	20	+0.59	+13.5¶	+13.8	37
1.71	Water	20	−0.06	−1.75¶	−1.4	11
1.31	"	20		−7.2	−6.8	31
1.98	"	20	−0.40	−10.1	−9.75	32, 33
2.24	"	20	−0.46	−10.35	−10.0	32, 33
2.21	"	20	−0.46	−10.42	−10.07	32, 33
2.03	"	21.2	−0.841	−10.5	−10.2	‖
2.22	"	20	−0.24	−10.8	−10.5	32, 33
	"	30		−10.35	−10.71	12
2.043	"	25.0	−0.865	−10.58	−10.58	This paper

* All preparations of leucine were isolated from protein sources with the exceptions noted.

† The *l*(−)-leucine was prepared by resolution of formyl-*dl*-leucine by the method of Fischer and Warburg (29).

TABLE II—*Concluded*

‡ The $l(-)$-leucine was prepared by resolution of formyl-*dl*-leucine with brucine; the *dl*-leucine was prepared by racemization of natural leucine and by synthesis from isovaleraldehyde but it was not stated which type of *dl*-leucine was resolved.

§ The $l(-)$-leucine was prepared by resolution of benzoyl-*dl*-leucine with quinidine; however, it was not stated whether racemized or synthetic *dl*-leucine was resolved.

‖ Stoddard, M. P., and Dunn, M. S., unpublished data.

¶ The specific rotation is abnormally low and presumably erroneous.

purified leucine exhibits a negative specific rotation in water which is definitely larger, and a positive specific rotation in acid which is definitely smaller, than the values reported in the literature (Table II) for other preparations of this amino acid. The coefficients employed in calculating the specific rotations at 25°, corresponding to the values in the literature, at other temperatures, were derived by the present authors in unpublished experiments. For the temperature range 15–25°, the coefficient for water is +0.072 and the coefficients for hydrochloric acid are +0.045 (12 N), +0.058 (7 N), +0.063 (6 N), +0.070 (5 N), +0.076 (4 N), +0.084 (3 N), +0.092 (2 N), and +0.11 (1 N). It was determined that the coefficients are altered insignificantly by variations in the concentration of $l(-)$-leucine ranging from 0.26 to 4.69 gm. per 100 ml. of 4.5 N hydrochloric acid solution and from 2 to 4 gm. per 100 ml. of 0.38 N hydrochloric acid solution.

It may be seen from the solubility data given in Table III that the values at 25° given in the literature are from about 0.5 to 12 per cent higher than the figure 2.19, obtained by the present authors. Solubilities at other temperatures, except in two cases, appear also to be too high and to deviate even further from the true values. These observations point significantly to the high purity of the authors' $l(-)$-leucine. In the investigations of Hlynka (46), which strengthen this view, it was found that the solubility of $l(-)$-leucine is 2.20 gm. per 100 gm. of water at 25°. The material employed in making this measurement was purified by S. W. Fox by fractional crystallization of the formyl derivative. The identical solubility was found, also, for the $d(+)$-leucine obtained by resolution of synthetic *dl*-leucine.

It may be concluded, from a consideration of the figures given in

Tables II and III, that a sample of relatively impure $l(-)$-leucine may be shown to be impure either by solubility or specific rotation data. On the other hand, as shown by the values reported by Takahashi and Yaginuma (12), attainment of the correct

TABLE III

Solubility of $l(-)$-Leucine in Water

Temperature	Solubility per 100 gm. water		Bibliographic reference No.	Temperature	Solubility per 100 gm. water		Bibliographic reference No.
	Observed	Corrected*			Observed	Corrected*	
°C.	gm.	gm.		°C.	gm.	gm.	
0.0	2.21	2.12	12	19	2.18	2.17	41
0	2.27†	2.12	34	19.5	2.24	2.17	42
5	2.28†	2.13	34	20	2.22	2.17	32, 33
10	2.30†	2.14	34	20	2.29	2.17	32, 33
13	2.44	2.15	39	20	2.37†	2.17	34
14.5	2.35	2.16	40	21	2.27	2.17	44
15	2.33†	2.16	34	22	2.44	2.18	45
16	2.41	2.16	41	25.0	2.28	2.19	12
16.5	2.53	2.16	24	25	2.47‡	2.19	7
17	2.22	2.16	42	25	2.29‡	2.19	7
17.5	2.61	2.16	43	25	2.43†	2.19	34
18	2.16	2.17	42	25	2.42§	2.19	26
18.5	1.99	2.17	11	25	2.20	2.19	46
19	2.24	2.17	42	25.1	2.19	2.19	This paper
19.0	2.27	2.17	12				

* Estimated on the basis of the present authors' value at 25.1° and the data given by Takahashi and Yaginuma (38).

† The specific rotation, $[\alpha]_D^{20}$ = +15.7° or $[\alpha]_D^{25}$ = +16.0° in 6 N HCl, indicates that this product was somewhat impure.

‡ The specific rotation, $[\alpha]_D^{25}$ = +15.7° in 6 N HCl, indicates that this product was somewhat impure.

§ The specific rotation, $[\alpha]_D^{18}$ = +17.75° or $[\alpha]_D^{25}$ = +18.4° in 6 N HCl, indicates that this product was impure. Solubility was determined at the isoelectric point.

specific rotation does not constitute proof that a given sample of $l(-)$-leucine is analytically pure. It would seem advisable for all naturally occurring amino acids, and perhaps obligatory in the case of leucine, that satisfactory analyses both of solubility and of specific rotation in water and acid be obtained before con-

cluding that the amino acid is analytically pure. In view of the difficulty, if not impossibility, of obtaining proof of absolute purity of such an amino acid, it may be recommended in cases in which even traces of impurities may be objectionable that additional analyses of the types described in the present paper be made if the purity of the amino acid is to be established with minimum uncertainty. On the other hand, it may be found that correct solubility values obtained under conditions such as those described herewith may be sufficient evidence of the analytical purity of a natural amino acid; however, this assumption has not been verified by experiment.

SUMMARY

It has been shown that *l*(−)-leucine of high purity may be prepared by recrystallization of crude natural leucine, as the monohydrochloride, from hydrochloric acid. The solubility of *l*(−)-leucine in water at 25.10° ± 0.03° is 2.19 ± 0.01 gm. per 100 gm. of water. The specific rotation of *l*(−)-leucine (*c* 2, *t* 25°, *l* 4, and λ 5893 Å.) is +15.20° ± 0.04° in 6 N hydrochloric acid and −10.57° ± 0.04° in water.

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THE SYNTHESIS OF SOME METHYLATED FATTY ACIDS

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Anderson and his associates have isolated a group of saturated, liquid fatty acids from the lipid fraction of tubercle bacilli and other acid-fast bacteria (1). The most interesting of the series is phthioic acid, which, injected into normal animals, stimulates cell proliferation with the formation of tubercular tissue. Tuberculostearic acid ($C_{19}H_{38}O_2$) is an innocuous substance with which it occurs and has been shown by Spielman (2) to be 10-methylstearic acid.

The structure of phthioic acid is still unknown. On the basis of a careful determination of the empirical formula ($C_{26}H_{52}O_2$) and a number of degradation experiments Spielman and Anderson (3) expressed the opinion that the molecule is formed from a long chain of about 22 carbon atoms with the remaining carbon atoms distributed laterally as methyl groups. To account for the high molecular rotation, one methyl group was believed to be in the α position and the others farther down the chain. The concept of phthioic acid as a methylated fatty acid was derived more from analogy with tuberculostearic acid than from concrete evidence.

Recently, Stenhagen and Ställberg (4), from a study of purely physical measurements, developed a strong case for a radically different formula; *viz.*, a trisubstituted acetic acid bearing one short chain and two long ones of unequal length. They present a thorough review of the pertinent facts which need not be repeated here. It suffices to say that the chemical evidence of Spielman and Anderson is fairly consistent with the new formula except for the isolation, as an oxidation product, of a steam-volatile C_{11}

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acid not identical with *n*-undecoic acid. The European authors suggest that this may have been a mixture of two or more acids. More serious is the identification by Wagner-Jauregg (5) of azelaic acid among the oxidation products. The facts become reconcilable, however, if the Stenhagen and Ställberg formula is modified so that at least one of the long chains is methylated or otherwise branched.

It is not likely that the problem will soon be solved by methods of degradation, and the synthetic approach offers the promptest returns. To determine the effect of methyl groups upon the physical and physiological properties of higher fatty acids we have synthesized several members of two series. The first series consists of α-methyl acids with even numbers of carbon atoms in the fundamental chain from α-methylstearic acid through α-methylhexacosanoic acid. The second series consists of 10-methyldocosanoic, 10-methyltetracosanoic, and 10-methylhexacosanoic acids.

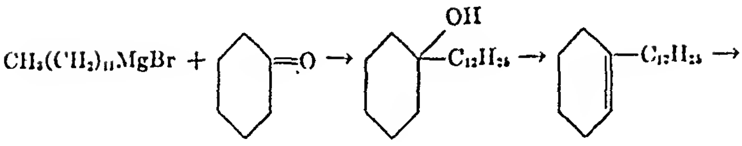
Synthetic Methods

Synthetic work in the field of higher fatty acids involves the lengthening of carbon chains, for only those chains of 18 or less carbon atoms are readily obtained. Methods now available suffer from either of two faults; they are too slow and laborious (malonic ester syntheses) or give too small yields as in the Robinson synthesis (6). A simple and rapid method of producing long chains in quantity would be of value. Several procedures were studied; one proved acceptable; two others were of but passing interest.

In an attempt to obtain long chain alcohols as starting materials, Kuhn's work (7) on the polymerization of crotonaldehyde was extended. Morpholine acetate added to redistilled crotonaldehyde gave a reddish brown syrupy mixture of polymers which hydrogenated readily over Raney's nickel. From the reduced product only *n*-octyl and lauryl alcohols could be profitably obtained.

$$CH_3CH{=}CHCHO \rightarrow CH_3(CH{=}CH)_nCHO \rightarrow CH_3(CH_2CH_2)_nCH_2OH$$

A second procedure studied was the synthesis of cyclohexenes, their oxidation to ketonic acids, and reduction to the corresponding fatty acids for later conversion to alkyl halides. The model used was 1-dodecylcyclohexene in the accompanying scheme.



$$C_{12}H_{25}CO(CH_2)_4COOH \rightarrow C_{17}H_{35}COOH$$

The alkylcyclohexene was prepared in 43 per cent yield, and oxidation with chromic acid gave 43 per cent of 6-ketostearic acid. Alkaline potassium permanganate oxidation led to 23 per cent yields. The ketonic acid was reduced quantitatively by the Clemmensen procedure. This method lengthened the chain by 6 carbon atoms with an over-all yield of about 18 per cent.

A third and most successful means was based upon the work of Blaise and Koehler (8) and others (2, 9). We were able to obtain

$$RZnCl + ClCO(CH_2)_8COOC_2H_5 \rightarrow RCO(CH_2)_8COOC_2H_5$$

10-ketodocosanoic acid and 10-ketotetracosanoic acid in 62 per cent yield of recrystallized acid. The reagents were readily prepared, and purification of the product was easy. Reduction to the saturated acids according to the conventional Clemmensen procedure was slow and incomplete, but a slight modification in which the ketonic acid and amalgamated zinc were refluxed in dry ethanol saturated with hydrogen chloride gave 85 to 88 per cent yields after 36 hours boiling. The acids (as esters) were hydrogenated to alcohols and converted to alkyl halides by the usual means.

Five α-methyl acids were prepared by using ethyl methylmalonate and alkyl iodides

$$CH_3CH(COOC_2H_5)_2 + RI \rightarrow RC(CH_3)(COOC_2H_5)_2 \rightarrow RCH(CH_3)COOH$$

Three 10-methyl acids were synthesized by the action of Grignard's reagents on ethyl 10-ketoundecanoate, followed by dehydration and hydrogenation.

$$RMgBr + CH_3CO(CH_2)_8COOC_2H_5 \rightarrow R\overset{\displaystyle CH_3}{\underset{\displaystyle OH}{C}}(CH_2)_8COOC_2H_5 \xrightarrow[\text{dehydration}]{\text{hydrolysis}}$$

$$R\overset{\displaystyle CH_3}{C}{=}CH(CH_2)_7COOH \xrightarrow[Ni]{H_2} R\overset{\displaystyle CH_3}{CH}(CH_2)_8COOH$$

The use of keto esters was found to be superior to the barium salt method employed in the synthesis of tuberculostearic acid (2).

TABLE I

Physical Constants of Methylated Acids

Acid	M.p.	Formula	Calculated			Found		
			C	H	Neutral equivalent	C	H	Neutral equivalent
	°C.		per cent	per cent		per cent	per cent	
2-Methylstearic.....	54.5	$C_{19}H_{38}O_2$	76.5	12.8	298.6	76.6	13.1	300.7
2-Methyleicosanoic..	61.5-62.0	$C_{21}H_{42}O_2$	77.2	13.0	326.6	77.0	13.2	327.3
2-Methyldocosanoic	67.0-67.5	$C_{23}H_{46}O_2$	77.9	13.1	354.6	77.8	12.9	357.9
2-Methyltetracosanoic............	72.0-72.5	$C_{25}H_{50}O_2$	78.5	13.2	382.6	78.5	13.1	385.4
2-Methylhexacosanoic......	75.5-76.0	$C_{27}H_{54}O_2$	79.0	13.3	410.7	78.9	13.2	411.1
10-Methyldocosanoic	45.5-46.0	$C_{23}H_{46}O_2$	77.9	13.1	354.6	77.9	13.1	355.4
10-Methyltetracosanoic........	51.0	$C_{25}H_{50}O_2$	78.5	13.2	382.6	78.1	12.9	379.8
10-Methylhexacosanoic..........	54.0-55.0	$C_{27}H_{54}O_2$	79.0	13.3	410.7	79.0	13.2	414

TABLE II

Physical Constants of Amides

	M.p.	Formula	N, calculated	N, found
	°C.		per cent	per cent
2-Methylstearamide .	104.5	$C_{19}H_{39}NO$	4.71	4.90
2-Methyleicosanamide . .	108.0	$C_{21}H_{43}NO$	4.31	4.36
2-Methyldocosanamide ..	109 -109.5	$C_{23}H_{47}NO$	3.96	4.17
2-Methyltetracosanamide...	111.5	$C_{25}H_{51}NO$	3.68	3.67
2-Methylhexacosanamide	113.0	$C_{27}H_{55}NO$	3.41	3.34
10-Methyldocosanamide .	78 - 78.5	$C_{23}H_{47}NO$	3.96	4.04
10-Methyltetracosanamide	79.0- 79.5	$C_{25}H_{51}NO$	3.68	3.77
10-Methylhexacosanamide	81 - 81.5	$C_{27}H_{55}NO$	3.41	3.43

Yields of crude hydroxy acid were 40 to 50 per cent, but after exhaustive purification this was frequently reduced to 10 to 15 per cent. The most persistent impurity, unchanged 10-keto-

undecanoic acid, was best removed by distillation from the crude mixture after dehydration. Neutral fractions were removed by extraction of the potassium salt. Hydrogenation of the unsaturated acid was equally effective with platinum or Raney's nickel catalysts.

The physical constants of the acids prepared are given in Table I, and the amides of each in Table II. The α-methyl acids crystallize as small, white platelets, melting about 15° lower than the corresponding normal acids. They are very soluble in ether, ethyl acetate, benzene, and acetic acid, less so in acetone, petroleum ether (b.p. 60–68°), and ethyl alcohol. The amides form fine, hard, white crystals melting about 40–50° higher than the corresponding acids. The 10-methyl acids are a microcrystalline white powder from acetone and small shiny crystals from acetone-carbon tetrachloride, melting about 35° lower than the straight chain acids. The solubility in all solvents is slightly greater than for the α-methyl acids.

Of the acids prepared, only α-methylstearic acid has been previously recorded. It was synthesized by Morgan and Holmes (10) from α-bromostearic acid and methyl magnesium iodide. The recorded melting point of 58° is slightly higher than ours and not on the smooth curve formed by plotting the melting points of the other four acids.

The physiological properties of the acids will be studied in another laboratory. The chemical work is not being continued by us.

EXPERIMENTAL

1-Dodecylcyclohexene—A Grignard reagent was prepared under an atmosphere of nitrogen from 5.75 gm. of magnesium and 59 gm. of dodecyl bromide in 150 cc. of dry ether. A solution of 23.5 gm. of cyclohexanone in 50 cc. of ether was slowly added at room temperature; the mixture was refluxed gently for 30 minutes, and allowed to stand overnight. It was decomposed with dilute sulfuric acid and ice, and washed with dilute sulfuric acid, then with water. After removal of the ether, the residue was steam-distilled. The remaining oil was separated, and dehydrated by heating with 30 gm. of powdered anhydrous zinc chloride on a steam bath for 2 hours. Water was added, and the hydrocarbon

was extracted with ether, washed twice with water, and dried over anhydrous sodium sulfate. Distillation gave 25.6 gm. or 43 per cent of theory; b.p. 140–143° at 1.5 mm.; $n_D^{25} = 1.4648$.

Analysis—$C_{18}H_{34}$ (250).	Calculated.	C 86.3, H 13.7
	Found.	" 86.1, " 13.7

6-Ketostearic Acid (Lactarinic Acid)—The best of numerous runs made with chromic acid and potassium permanganate under varying conditions was the following. 5 gm. of chromic acid dissolved in 20 cc. of equal parts of acetic acid and water were added to 3.5 gm. of 1-dodecylcyclohexene in 80 cc. of 90 per cent acetic acid, and the mixture was warmed to 90° on a steam bath, taken off, and stirred vigorously for 2 hours. The solution was then poured into 600 cc. of cold water and filtered with suction. The solid was crystallized from absolute ethyl alcohol to give 1.8 gm. (43 per cent) of a powder faintly tinted with green, m.p. 83°. Recrystallization from ethyl alcohol and then from acetone gave shiny white flakes melting at 86.5–87° (11). A Clemmensen reduction yielded stearic acid, m.p. 68.5–69°.

10-Ketodocosanoic Acid—A Grignard reagent was prepared from 0.72 gm. of magnesium and 7.5 gm. of dodecyl bromide in 50 cc. of dry ether in a 500 cc. flask fitted with the usual equipment and an inlet through which nitrogen was continuously passed. To the reagent were added 4.1 gm. of freshly fused zinc chloride dissolved in 12 cc. of ether. After the mixture was refluxed for 30 minutes, 60 cc. of sodium-dried benzene were added to dissolve the white precipitate of dodecyl zinc chloride. With continuous stirring and refluxing, 6.0 gm. of ω-carbethoxynonoyl chloride (prepared from ethyl hydrogen sebacate and thionyl chloride in 90 per cent yield (12)) dissolved in 50 cc. of dry benzene were slowly added. The reaction mixture was refluxed for another 2 hours, decomposed with dilute hydrochloric acid, and washed with dilute ammonium chloride solution, water, dilute sodium carbonate, and water. After removal of the solvent the syrupy residue was saponified. The crystalline sodium salt was very insoluble in both cold water and cold benzene, and was purified by thorough extraction with each. The filtered salt was digested with dilute hydrochloric acid, and the free acid crystallized from acetone-petroleum ether (b.p. 60–68°) to give 5.3 gm. of light, shiny crystals. This is 62

per cent of theory. After one more crystallization the melting point was 91.5°.

Analysis—$C_{22}H_{42}O_3$ (354.6)

Calculated.	C 74.5, H 12.0, neutral equivalent	354.6
Found.	" 74.4, " 12.2, " "	356.6

n-Docosanoic Acid—Dry hydrogen chloride was passed into 100 cc. of 99.5 per cent ethyl alcohol until it was nearly saturated, and then 50 gm. of amalgamated zinc and 5 gm. of 10-ketodocosanoic acid were added and the mixture was gently refluxed for 16 hours. More hydrogen chloride was passed in and refluxing was continued for 20 hours. The solution was then poured into excess aqueous potassium hydroxide and refluxed 2 hours. A heavy white precipitate was removed from the cooled solution by filtration. The acid was liberated from the precipitate by prolonged digestion with dilute hydrochloric acid, and dissolved in hot petroleum ether (b.p. 100–140°). The hot solution was filtered and allowed to cool, to give 4.2 gm. (88 per cent) of fine white plates melting at 79–80.5° (13). There was no depression in melting point when the product was mixed with *n*-docosanoic acid obtained from natural sources.

10-Ketotetracosanoic Acid—This was prepared from tetradecyl magnesium bromide by exactly the same procedure in the same yield. The crystals melted at 94–94.5° after crystallization from petroleum ether (b.p. 60–68°).

Analysis—$C_{24}H_{46}O_3$ (382.6). Calculated. C 75.3, H 12.1
Found. " 75.5, " 12.1

n-Tetracosanoic Acid—This was made from 10-ketotetracosanoic acid in the same manner as *n*-docosanoic acid. It melted at 82.5–83.5° (13) and was identical with a sample obtained by the malonic ester synthesis.

α-Methyl Acids—These were all synthesized in the same manner. The alkyl iodides were prepared from the alcohols by the general method of refluxing the alcohol with iodine and red phosphorus (14). The alcohols in turn were obtained by reduction of the corresponding esters.

The procedure for α-methylstearic acid is representative. 13 gm. of diethyl methylmalonate (15) were added to the product of

reaction between 1.4 gm. of clean sodium and 30 cc. of dry n-butanol. The solution was warmed for 10 minutes, and 10.3 gm. of cetyl iodide were added. The mixture was refluxed for $3\frac{1}{2}$ hours, cooled, and ether and water added. The ether layer was washed with water, very dilute sodium thiosulfate (an emulsion readily forms), and finally water again. The ether was removed and the residue saponified. The dicarboxylic acid obtained after acidification was heated under 10 mm. pressure to 150–180° for 3 hours. The straw-colored residue weighed 7.6 gm. (88 per cent of theory) and melted at 53.5–55°. Two crystallizations from petroleum ether (b.p. 60–68°) gave fine white flakes melting at 54.5°.

The acids were crystallized from petroleum ether, or acetone-petroleum ether. The yields of crude acid after decarboxylation were 81 to 88 per cent of theory. After one crystallization the yields were 69 to 75 per cent. Amides were prepared in about 50 per cent yield by refluxing with thionyl chloride and pouring into cold concentrated ammonia. They were crystallized two or three times from methyl alcohol.

10-Ketoundecanoic Acid—This was prepared from methyl zinc iodide and ω-carbethoxynonoyl chloride in essentially the same way as that used by Ruzicka and Stoll (12). In a large number of attempts our best yield of acid was 43 per cent, but the average was 30 per cent. The yield of crude ester was higher, 40 to 55 per cent, but upon saponification much sebacic acid was invariably obtained. The acid was readily reconverted to the ethyl ester boiling at 153–154° at 6 mm.; $n_D^{25} = 1.4384$.

10-Methyl Acids—The method found to be of most value was the following. A Grignard reagent was prepared from 1.37 gm. of magnesium and 15.8 gm. of tetradecyl bromide in 100 cc. of dry ether under an atmosphere of nitrogen. The condenser outlet was then corked, the separatory funnel stem pushed to the bottom of the flask, and the stop-cock opened so that the nitrogen pressure forced the solution into the funnel. The flask was rinsed with 20 cc. of ether added through the condenser and forced into the funnel. 10 gm. of ethyl 10-ketoundecanoate in 100 cc. of ether were placed in the flask, maintained at 23°, and the Grignard reagent was slowly added (1 hour). The mixture was stirred at 25° for 3 hours, then refluxed for 2 hours more, and allowed to stand overnight.

After decomposition with dilute hydrochloric acid, and washing

with water, dilute sodium carbonate solution, and water, the ether was removed and the residue saponified. An insoluble top layer of *n*-octacosane resulting from coupling was removed from the alkaline solution. The solution was acidified, and the acid obtained was heated to 180–210° with a trace of iodine, to eliminate about 3 cc. of water. The residue was distilled under reduced pressure. A fraction boiling below 165° at 1 mm. was collected, and then 1.1 gm. at 166–170° at 1 mm. This fraction melted at 57.5° after crystallization from petroleum ether. (10-Ketoundecanoic acid boils at 166–167° at 1 mm. (16) and melts at 58.5–59.5°.) The undistilled portion of 12.4 gm. was dissolved in ether and extracted with aqueous potassium hydroxide. This removed 2.5 gm. of a waxy neutral fraction. The remaining acid was treated with norit, dissolved in 75 cc. of 99 per cent ethyl alcohol, and hydrogenated over 2 gm. of Raney's nickel catalyst at 175° under a pressure of 160 atmospheres of hydrogen. The solution was filtered, saponified, and the acid obtained in the usual manner. A yield of 5.71 gm. of acid after crystallization from acetone melted at 49.5–51°. This was a yield of 34 per cent. Two recrystallizations from acetone gave a fine white powder melting at 51–51.5°.

The other acids were obtained in a similar manner. Amides were prepared in slightly poorer yields than with the α-methyl acids, and crystallized from acetone or methyl alcohol.

SUMMARY

A method has been developed for the synthesis of higher fatty acids.

The α-methyl acids with even-numbered fundamental carbon chains from α-methylstearic to α-methylhexacosanoic acid have been synthesized. 10-Methyldocosanoic, 10-methyltetracosanoic, and 10-methylhexacosanoic acids were also made. The corresponding amides were prepared as derivatives.

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STARCH PHOSPHORYLASE OF POTATO

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Cori and his colleagues (1–6) discovered and elucidated the series of reversible chemical reactions by which glucose is transformed into glycogen in animal tissues. These reactions are catalyzed by a group of enzymes, the most fundamental of which is phosphorylase, the enzyme which catalyzes the condensation of glucose-1-phosphate (Cori ester) to starch or glycogen. The properties of phosphorylase from animal sources have been systematically investigated by Cori *et al.* (1–5), while Hanes (7, 8) discovered a similar enzyme in extracts from pea and potato, and made a thorough study of the crude potato enzyme. The present investigation deals with the preparation and some of the characteristics of purified potato phosphorylase.

Method of Testing and Preparation

1 unit of phosphorylase activity has been defined as the amount of enzyme which catalyzes the liberation of 0.1 mg. of inorganic phosphate from glucose-1-phosphate in 3 minutes at 38° and pH 6.0. The test mixture consisted of 5 to 10 units of enzyme, 0.5 cc. of 0.5 M citrate buffer of pH 6.0, 0.2 cc. of 5 per cent soluble starch, and 1 cc. of 0.1 M glucose-1-phosphate, the final volume being 3.5 cc. The glucose-1-phosphate was added to the rest of the mixture after temperature equilibration. The reaction was stopped at some convenient time (5 to 10 minutes) by addition of 5 cc. of 5 per cent trichloroacetic acid and 2 cc. of 2.5 per cent ammonium molybdate[1]

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[1] When ammonium molybdate reagent was added according to the Fiske and Subbarow phosphate procedure, our test solutions always became turbid and made accurate colorimetric readings impossible. Addition of

in 5 N sulfuric acid. The mixture was diluted to 25 cc. with water, and an aliquot of the filtrate was analyzed by the method of Fiske and Subbarow (9). Fig. 1 shows that under the above experimental conditions the activity is directly proportional to the concentration of the enzyme.

The crystalline dipotassium salt of glucose-1-phosphate was prepared according to the method of Hanes (8). The α_D of the preparation was +77° (+78° according to Hanes (8)). The ester contained no inorganic P and 8.8 per cent of esterified P which was completely hydrolyzable in 5 minutes in N HCl at 100°.

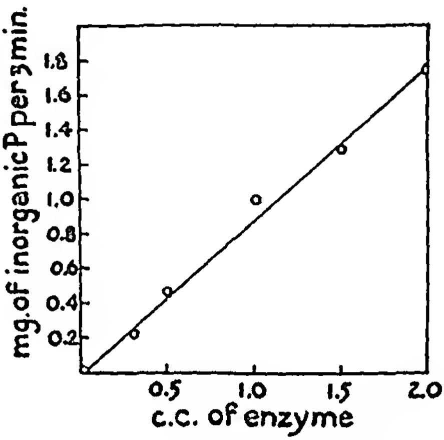

FIG. 1. Variation of activity with concentration of enzyme

The ratio (ϵ 280 mμ)/(enzyme units) which we shall refer to as the activity ratio (10) was used as an index of the purity level of the enzyme throughout the purification procedure.

Potato phosphorylase was prepared as follows: 4.5 kilos of store potatoes were peeled, sliced, and immersed in 5 liters of 0.005 M KCN for 2 hours. The slices, after removal, were minced, and the juice pressed out in a screw press (volume 1.76 liters). The juice was rapidly heated to 50° and maintained at that temperature for 5 minutes. While the coagulated suspension was still warm,

the molybdate reagent to our solutions immediately after trichloroacetic acid addition and before filtration of the suspension prevented subsequent turbidity. The molybdate concentrations in the phosphate estimations were then brought to the same level as those in the Fiske and Subbarow method.

20 gm. of ammonium sulfate were added for each 100 cc. of fluid. The precipitate was filtered off and discarded. To the clear filtrate were added 15 gm. of ammonium sulfate per 100 cc. of fluid. The precipitate was centrifuged hard and dissolved in 100 cc. of 0.25 M citrate buffer of pH 6.0. The yields of the enzyme are shown in Table I.

The subsequent purification involved four successive series of fractionation with ammonium sulfate, the details of which are summarized in Table I. The fractions retained were Fractions II and III in the first fractionation, Fractions B and C in the second,

TABLE I

Results of Fractionations with Ammonium Sulfate

Fraction No.	Degree of saturation	Total enzyme units	Activity ratio
I	0.35	470	1.41
II	0.39	2260	0.84
III	0.44	1520	0.86
IV	0.51	135	4.00
A	0.38	680	0.94
B	0.41	1470	0.70
C	0.45	1220	0.71
D	0.52	100	
a	0.37	690	0.46
b	0.42	2440	0.36
c	0.47	520	
γ	0.42	2425	0.30

and Fraction b in the third. Careful fractionation of Fraction b yielded our preparation of highest purity.

From a total of 4400 enzyme units in 1.76 liters of potato juice, the final yield was 2425 enzyme units of activity ratio 0.30 in 20 cc. Since 1 unit in the original potato was equivalent to 120 mg. of dry weight, whereas 1 unit of the final preparation was equivalent to 0.324 mg. of dry weight, the degree of concentration was 120/0.324 or 370. The ratio of protein to carbohydrate in the potato is about 1:8. The degree of concentration in terms of protein was therefore about 46.

Other methods of purification were explored without success. Alumina absorption was not found effective in the purification of potato phosphorylase. Only about 35 to 40 per cent of the total

enzyme adsorbed could be recovered with various eluting agents. Dialysis for 12 hours against running tap water led to small losses. The enzyme was inactivated after prolonged dialysis against distilled water but not after prolonged dialysis against 0.2 M KCl. Precipitation in the cold with alcohol inactivated the enzyme completely. The enzyme was stable at 0° in dilute ammonium sulfate solution (about pH 6 to 7) for some days; but steady though slow deterioration of activity was observed over a period of weeks. Activity rapidly declined when the enzyme was exposed to solutions the pH of which was less than 5 or greater than 9. Stability and activity both were maximum at about pH 6.

The final preparation was always brown in color. Ultracentrifuge results showed that the substance responsible for the color was not associated with the protein components of the preparation and was apparently of low molecular weight. The colored component could be reduced with $Na_2S_2O_4$ to a light yellow substance (non-oxidizable) without affecting the activity of the enzyme. There was no evidence of any functional relationship between the amount of color and the enzymic activity of different preparations.

Samples of our best preparations (activity ratio 0.3) were examined by Dr. Oncley in the ultracentrifuge. Two components were found present in the ratio of 7:3 with sedimentation constants ($S_{20} \times 10^{13}$) of 5.5 to 5.7 and 2.3 to 2.5 respectively. Until the purification is advanced a stage further, it cannot be decided which of the two components is identical with the enzyme.

At the 0.3 activity ratio level of purity, 1 mg. of protein is equivalent to 3.08 enzyme units. Thus, 1 mg. of protein under the conditions of the test catalyzes the formation of 6.16 mg. of inorganic P per hour, which corresponds to the formation of 32 mg. of starch in 1 hour at 38°.

Components of Catalytic System—There are three components necessary for the catalytic condensation of glucose-1-phosphate to starch: (1) enzyme, (2) glucose-1-phosphate, and (3) catalytic amounts of a suitable polysaccharide, *e.g.* starch, dextrin, or glycogen. The animal enzyme has been shown by Cori and Cori (4) to be a highly dissociated complex of specific protein and adenylic acid. However, the activity of the potato enzyme is neither dependent upon nor influenced by the presence of adenylic acid. The possibility was examined that the potato enzyme was a

firmly conjugated compound of specific protein and adenylic acid which was not resolved in the course of the purification procedure. This was tested with the animal enzyme by using boiled or acid-hydrolyzed preparations of potato enzyme in concentrated form as a source of adenylic acid. The results of these experiments showed no evidence from this direction that adenylic acid is present in potato phosphorylase.

A mixture of glucose-1-phosphate (dextrin-free) and the enzyme does not yield starch. On addition of catalytic amounts of starch, dextrin,[2] or glycogen, synthesis of starch with simultaneous liberation of inorganic P ensues. The necessity for added polysaccharide is obscured when a sample of naturally prepared glucose-1-phosphate is tested. Traces of catalytically active dextrins are invariably present and these are not removed completely by successive crystallizations. Thus we have never been able to demonstrate a perfect blank in absence of added polysaccharide, using naturally prepared glucose-1-phosphate. Through the kindness of Dr. C. Cori, we were able to test a sample of synthetic glucose-1-phosphate prepared by the method of Cori *et al.* (11). The evidence was clear (*cf.* Table II) that in complete absence of polysaccharide, glucose-1-phosphate was not condensed to starch by the potato phosphorylase.

Specificity of Components—Our experiments confirm the observations of Hanes (8) that glucose-6-phosphate, glucose, fructose, fructose-1-phosphate, and fructose-1:6-phosphate cannot replace glucose-1-phosphate as substrate.

Starch was the most active polysaccharide catalyst for potato phosphorylase. Dextrin was slightly less active, glycogen showed 77 per cent of the activity of starch, while sucrose, glucose, the Schardinger dextrins, and maltose were inactive.

The form or the origin of the starch employed appeared to be immaterial. Corn, rice, arrow-root, and wheat starches were as effective as potato starch. Similarly, soluble as well as insoluble amyloses were interchangeable. Samples of highly purified corn-starch and glycogen showed no decrease in catalytic activity compared to the corresponding crude products. Furthermore, when starch was treated with diastase for a period sufficient to eliminate

[2] Merck's dextrin reagent.

staining power with iodine, the catalytic power of the hydrolysate also disappeared. Catalytic action cannot, therefore, be ascribed to a non-polyhexose impurity.

Hanes (8) has reported that maltose in high concentration shows some activity as a catalyst. While our experiments with crude

TABLE II

Effect of Polysaccharide Catalyst on Starch Formation

Each test mixture consisted of 1 cc. of enzyme, 0.5 cc. of 0.5 M citrate buffer of pH 6, and 1 cc. of 0.1 M glucose-1-phosphate.

Additions	Inorganic P per 3 min.
	mg.
Synthetic glucose-1-phosphate	0.00
" " + 12.5 mg. soluble starch	1.14
Glucose-1-phosphate isolated from potato juice	0.08
" " " " " + 12.5 mg. soluble starch	1.15

TABLE III

Decrease in Catalytic Effect with Purification of Maltose

Each test mixture consisted of 1 cc. of enzyme (8 units), 1 cc. of 0.1 M dextrin-free glucose-1-phosphate, and 0.5 cc. of 0.5 M citrate buffer of pH 6.0.

Polysaccharide catalyst	Catalytic effect, mg. inorganic P per 3 min.
None	0.00
5 mg. starch	0.80
50 " commercial maltose	0.67
50 " once recrystallized maltose	0.35
50 " twice " "	0.18

maltose confirmed this observation, recrystallization of maltose preparations whittled the catalytic effect down to the vanishing point (*cf.* Table III). Here again, the presence of catalytic amounts of dextrin impurity had to be reckoned with. This impurity was not completely eliminated after two successive recrystallizations of maltose.

Factors Affecting Activity of Enzyme—The effects of variation of

hydrogen ion concentration, temperature, concentration of glucose-1-phosphate, and concentration of polysaccharide catalyst are shown in Figs. 2 to 4. The potato enzyme was rapidly inactivated at temperatures above 58°. Thus, exposure for 3 minutes at 58° led to a loss of 61 per cent of the activity, and for 3 minutes at 68° to a loss of 97 per cent.

The effects of special reagents and inhibitors on the activity of potato phosphorylase are shown in Table IV. Copper and phlor-

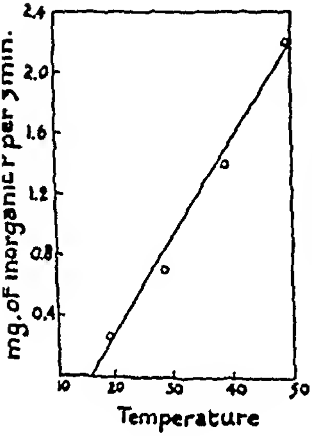

Fig. 2

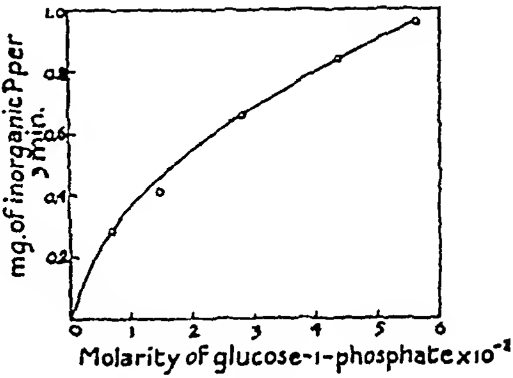

Fig. 3

Fig. 2. Variation of activity with temperature.
Fig. 3. Variation of activity with concentration of glucose-1-phosphate.

hizin which inhibit strongly the activity of the muscle enzyme showed little or no effect on the potato enzyme.

The non-reducing and non-fermentable Schardinger dextrins[3] which are produced by the action of *Bacillus macerans* on starch paste showed the phenomenon of competitive inhibition of the enzyme (Table V). Increasing the concentrations of the α- or β-dextrins for a given concentration of starch catalyst inhibited proportionately the activity of the enzyme. Increasing the concentration of starch catalyst for a given concentration of α- or β-dextrin decreased proportionately the inhibitory action of the

[3] Purified Schardinger dextrins were obtained from Dr. T. Schoch of the Corn Products Refining Company of New York.

Schardinger dextrins. It would appear that the Schardinger dextrins and starch were competing for the same active group in the

TABLE IV

Effect of Various Reagents on Activity of Enzyme

Reagent	Final concentration	Inhibition
		per cent
$AgNO_3$	M/50,000	8
"	M/10,000	80
$CuSO_4$	M/50,000	0
"	M/10,000	0
"	M/1,000	0
$ZnSO_4$	M/25,000	0
$HgCl_2$	M/50,000	0
$PbAc_2$	M/25,000	0
Iodoacetic acid	M/27	0
Sodium fluoride	M/27	24
Phlorhizin	0.7 saturated	18
"	0.3 "	0
Glucose	M/27	0
Potassium cyanide	M/27	0
Capryl alcohol	Saturated solution	0
Sulfanilamide	5.3 mg. %	0
Hydrogen peroxide	1%	0
$Na_2S_2O_4$	53 mg. %	0

TABLE V

Inhibition by Schardinger Dextrins

Each reaction mixture consisted of 1 cc. of enzyme (12 units), 1 cc. of 0.1 M glucose-1-phosphate, and 0.5 cc. of 0.5 M citrate buffer of pH 6.0.

Dextrin	Soluble starch	Inhibition
mg.	*mg.*	*per cent*
15	12.5	23
25	12.5	52
50	12.5	73
50	12.5	73
50	25.0	50
50	50.0	21

enzyme. This inhibitory action was not shown by inulin, sucrose, or maltose in a similar range of concentration.

Properties of Starch Formed in Reaction—A mixture of glucose-

1-phosphate, enzyme, and an amount of polysaccharide catalyst just sufficient to initiate the reaction produced starch at a linear rate which was but a small fraction of the maximum velocity in presence of excess polysaccharide catalyst (*cf.* Fig. 5). The dilemma arose that if starch was formed in the reaction the rate of formation of starch should have increased autocatalytically until the maximum rate was attained. Since there was no indication of this autocatalytic form of the time-velocity curve, it was concluded

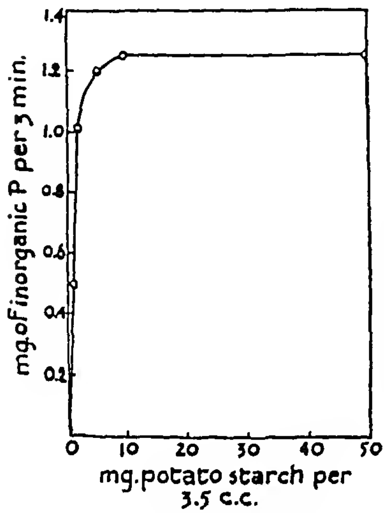

FIG. 4

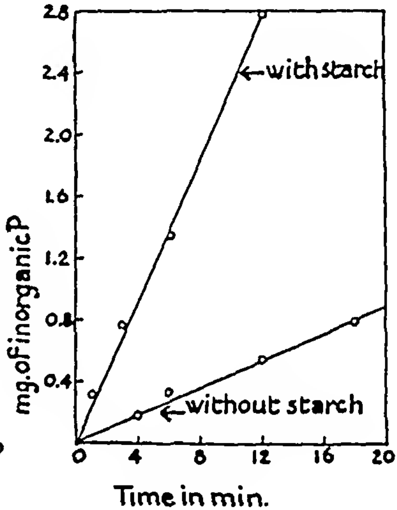

FIG. 5

FIG. 4. Variation of activity with concentration of starch as polyhexose catalyst.

FIG. 5. Time-activity curves for reactions in presence and in absence of added polyhexose catalyst.

that the starch formed in the reaction, unlike the other starches tested, could not act as a catalyst for its own formation. This non-catalytic property of the starch formed by the potato enzyme system was confirmed by direct experiment. The enzymatically formed starch was sparingly soluble in water and rapidly retrograded. It precipitated out in a concentrated solution and could be centrifuged off and washed (*cf.* Hanes (8) for a study of its physical and chemical properties). The aqueous solution or suspension of this starch when added to the polysaccharide-free system did not catalyze the further formation of starch (*cf.* Table

VI). It would be of considerable interest to know the chemical basis for this difference in catalytic properties between reagent starch and the starch formed as the reaction product of phosphorylase action. Comparison of iodine staining power (8), x-ray diagrams (6, 12), and elementary analysis (8) have failed thus far to pick out any significant differences.

TABLE VI

Synthesized Starch As Catalyst

Each reaction mixture consisted of 1 cc. of enzyme, 1 cc. of 0.1 M glucose-1-phosphate, and 0.5 cc. of 0.5 M citrate buffer of pH 6.0.

	Catalytic effect, mg. inorganic P per 3 min.
Starch synthesized by potato phosphorylase (5 mg.)	0.00
Soluble potato starch (5 mg.)	0.64
Insoluble potato starch (5 mg.)	0.64

TABLE VII

Components of Lima Bean Phosphorylase System

Each reaction mixture consisted of 0.5 cc. of enzyme and 0.5 cc. of 0.5 M citrate buffer at pH 6.0.

System	Inorganic P per 3 min.
	mg.
Enzyme	0.00
" + glucose-1-phosphate	0.62
" + " + soluble starch	0.67
" + " + dextrin	0.63
" + " + soluble starch + adenylic acid	0.65

Rôle of Polysaccharide Catalyst—The polyhexoses that are catalytically active in starch formation have two characteristics in common: (*a*) they are all composed of chains of α-glucose units in 1:4 glucosidic linkage, and (*b*) they can all be phosphorylyzed to glucose-1-phosphate in presence of inorganic phosphate and the enzyme. The chain length appears to be of importance. Thus α and β Schardinger dextrins with probable chain lengths of four and six respectively are inactive, whereas the violet-staining dextrins of between six and twelve are fully active. The fact that

a polyhexose catalyst must be capable of being phosphorylated may bear some relation to its catalytic rôle in starch formation. Possibly the initial step in starch formation is the transfer of phosphate from glucose-1-phosphate to the catalyst. Inorganic phosphate would then arise by the spontaneous decomposition of the catalyst-phosphate compound.

The starch phosphorylase enzyme of Lima bean has been prepared by the same method as that employed in the purification of the potato enzyme, and has been brought to a comparable degree of purity. Only two components were necessary for starch formation, (*a*) the enzyme and (*b*) glucose-1-phosphate (*cf.* Table VII). Addition of starch, dextrin, or glycogen did not increase the rate of condensation of glucose-1-phosphate to starch. This result cannot be explained in terms of some polysaccharide contaminating the enzyme, as is shown by the fact that the preparation of the Lima bean enzyme did not act as a source of polyhexose catalyst for the potato enzyme. There are two possible explanations: either the polyhexose catalyst is an integral part of the Lima bean enzyme molecule or the mechanism of the reaction is different, and does not depend upon the collaboration of a polyhexose.

SUMMARY

The purification and some of the properties of potato phosphorylase are described. The enzyme was concentrated some 370 times by a series of successive ammonium sulfate fractionations. Adenylic acid is not a component of the system, although catalytic amounts of starch, dextrin, or glycogen are required for starch formation from glucose-1-phosphate. Some of the factors affecting starch formation by the enzyme are analyzed.

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THE GONADOTROPIC HORMONE OF URINE OF PREGNANCY. IV

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The gonadotropic hormone from the urine of pregnant women has been isolated in highly purified form (1) and studied by means of chemical and physical methods (2). In order to characterize it more exactly, further studies have been made by means of the ultracentrifuge and the Lamm diffusion apparatus. In addition, more convincing evidence concerning the molecular weight has been obtained by an estimation of the partial specific volume. Since the hormone is a glycoprotein, it was thought unjustifiable to assume the value of 0.70 to 0.75, which has been found characteristic for most proteins.

The procedure of isolation (previously reported (1)) yielded such a highly purified hormone preparation that no further purification was undertaken of the material used in the following studies. The material assayed 4000 minimal effective doses (Friedman) or about 8000 international units per mg.

Molecular Kinetic Behavior—Sedimentation velocity and diffusion experiments were performed in a phosphate-borate buffer medium of pH 7. The solution, containing 8.2 mg. of protein per ml. of solvent, was made up to be 1 per cent in sodium chloride. Three preliminary and two complete and precise sedimentation velocity experiments were carried out in a standard Svedberg oil turbine ultracentrifuge operating at 60,000 R.P.M. Observations of the position of the boundary in the cell at the end of definite intervals of time were made by the Lamm scale method. The operation of the ultracentrifuge and the evaluation of the sedimen-

tation velocity constant have been described in detail (3). The symbols used by Svedberg and Pedersen have been retained throughout.

In Experiment 4 observations of the position of the boundary in the cell were made over a period of $2\frac{1}{2}$ hours. The average sedimentation constant, corrected to a process taking place in water at 20° and calculated from the position of the boundary at eight successive exposures after 15 minute intervals, was $s_{20} = 4.3 \times 10^{-13}$ cm. per second per unit field. The calculations and results of Experiment 5 are summarized in Table I.

TABLE I

Calculation of Sedimentation Constant of Urinary Gonadotropin

Protein concentration 8.2 mg. per ml.; optical magnification factor 0.595; speed 60,000 R.P.M.; time intervals between exposures 10 and 15 minutes.

Observation No.	$x_{0.5}$	Δx	x_m	$\omega^2 \times 10^{-5}$	Temperature	$\frac{\eta_t}{\eta_{20}}$	$s \times 10^{13}$
					°C.		
4	3.233			394.8	21.9	0.955	
5	3.333	0.059	5.857	394.8	23.0	0.930	3.96
6	3.495	0.063	5.918	394.8	23.6	0.918	4.12
7	3.596	0.061	5.980	394.8	24.0	0.910	3.92
8	3.745	0.070	6.045	394.8	24.4	0.902	4.41
9	3.839	0.059	6.110	394.8	24.8	0.894	3.64
10	4.005	0.101	6.190	394.8	25.3	0.884	4.06
11	4.097	0.103	6.292	394.8	25.6	0.878	4.04
12	4.240	0.084	6.385	394.8	26.0	0.870	3.22
13	4.415	0.106	6.480	394.8	26.2	0.866	3.99
14	4.586	0.100	6.583	394.8	26.4	0.862	3.69

$s_{av} = 3.90 \times 10^{-13}$.
$s_{20} = s_{av} \times 1.1 = 4.3 \times 10^{-13}$.

Two diffusion experiments were performed in a thermostat at 2°. The diffusion took place in one case in an arm of a Tiselius electrophoresis cell (4). In the other experiment the boundary was formed in the steel cell described by Lamm (5). The blurring of the boundary as a function of time was followed by the refractive index method of Wiener as modified by Lamm. Diffusion constants were calculated by the method of area and maximum height of the scale line displacement-distance curve. The results of the calculations for Experiment 2 are given in Table II.

Sedimentation velocity and diffusion scale line displacement-distance curves conform reasonably well to ideal distribution curves, indicating that the urinary gonadotropin as prepared is essentially monodisperse.

Partial Specific Volume—Compared with sedimentation data relatively few determinations of partial specific volume have been made. As the hormone is an unusual protein, a glycoprotein containing 15 per cent carbohydrate groups, it was felt that a determination of partial specific volume was essential for the calculation

TABLE II

Calculation of Diffusion Constant of Urinary Gonadotropin

Observation No.	Area	H_{max}	t	$\frac{A^2}{H^2_{max} \cdot t}$
	sq. cm.	*cm.*	*sec.*	
1	0.009876	0.0146	97,200	4.61×10^{-6}
2	0.009756	0.0112	147,600	5.14×10^{-6}
3	0.009408	0.0106	169,200	4.66×10^{-6}
4	0.009224	0.0098	197,100	4.50×10^{-6}
5	0.009348	0.0087	231,300	4.99×10^{-6}
6	0.009480	0.0086	251,100	4.78×10^{-6}

For each observation the value of $\left(\frac{l-b}{l}\right)^2 \frac{1}{4\pi G^2}$ was 4.76×10^{-2}.

$$D_t = \frac{A^2}{H^2 t}\left(\frac{l-b}{l}\right)^2 \frac{1}{4\pi G^2} = 2.28 \times 10^{-7}.$$

$$D_{20} = D_t \times \frac{\eta_t}{\eta_{H_2O}} \times \left(\frac{\eta_t}{\eta_{20}}\right)_{H_2O} \times \frac{293.1}{273.1} = 4.4 \times 10^{-7}.$$

of the molecular weight. Actually it was found that the partial specific volume was similar to that of most other proteins.

The partial specific volume of the hormone was determined by dissolving a weighed amount of it in water and making the solution up to a definite volume in a volumetric flask. In the determination the required datum is the weight of water displaced by a given weight of hormone. By determination with a pycnometer 0.0173 gm. of water was displaced by 0.0226 gm. of hormone, giving as value for the partial specific volume 0.76. There is a possible error in the determination of partial specific volume of approximately ±5 per cent.

Molecular Weight of Urinary Gonadotropin—According to the

formula, $M = RTs_{20}/(D_{20}(1 - V\rho))$, in which M is the molecular weight, V is the partial specific volume, and ρ is the density of water at 20°, the molecular weight of the hormone is calculated to be close to 100,000. As is evident from the formula, an error of ±5 per cent in the partial specific volume can produce a variation of ±15 per cent in the calculated molecular weight.

SUMMARY

A highly purified urinary gonadotropin preparation assaying 4000 minimal effective (Friedman) doses per mg. was found to be essentially monodisperse and yielded the following data.

The sedimentation constant, $s_{20} = 4.3 \times 10^{-13}$ cm. per second per unit field.

The diffusion constant, $D_{20} = 4.4 \times 10^{-7}$ sq. cm. per second.

The partial specific volume is 0.76.

The molecular weight is in the neighborhood of 100,000.

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METABOLISM OF LACTIC ACID CONTAINING RADIOACTIVE CARBON IN THE α OR β POSITION

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The fate of the labeled carboxyl carbon atom of lactic acid fed to fasted rats has been studied previously (1). The liver glycogen formed averaged 32 per cent of the lactate fed, but contained only 1.6 per cent of the radioactivity. These results might be interpreted as an effect of a "dilution" of the labeled, 3-carbon chain in the animal body. They do not necessarily indicate that the carboxyl carbon atom is detached from the rest of the carbon chain in the course of glycogen synthesis. However, a comparison of these results with analogous experiments employing lactate containing C^{11} in the α or β position (Type II lactate[1]) should show whether such a step does occur. Experiments with Type II lactate will be reported in this paper. The liver glycogen formed averaged 20 per cent of the lactate fed and contained 3.2 per cent of the radioactivity, a definitely higher amount than that previously found with Type I.

EXPERIMENTAL

The synthesis of Type II lactate was carried out according to the procedure of Cramer and Kistiakowsky (2) except that some modifications were introduced in the preparation of radioactive acetylene, $CH{\equiv}C^{11}H$. These will be described in detail.

The method employed by Ruben, Hassid, and Kamen (3) for

[1] For convenience and in accordance with previous usage (2), $CH_3CHOHC^{11}OOH$ will be designated Type I lactate; $CH_3C^{11}HOHCOOH$ and $C^{11}H_3CHOHCOOH$ will be called Type II lactate.

removing C^{11} from the target was found superior to that previously employed, mainly because of the saving in time and the decreased exposure of the operator to radiation. The $C^{11}O_2$ was pumped from the chamber and absorbed in a liquid air trap containing 3 cc. of 2 M NaOH and 2.5 mM of Na_2CO_3 as carrier. The trap was evacuated and immersed in boiling water until the contents had liquefied. A few minutes were allowed for absorption of CO_2. The carbonate was precipitated as $BaCO_3$ by the addition of 3 cc.

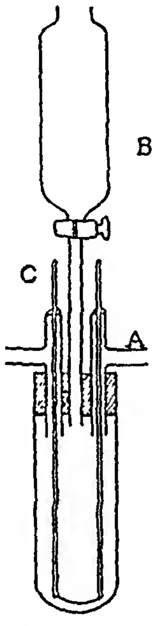

FIG. 1. Apparatus for the preparation of acetylene, consisting of a 50 cc. Pyrex centrifuge tube closed by a 3-hole rubber stopper; *B*, separatory funnel; *A*, T-tubes; *C*, iron electrodes.

of 1 M $BaCl_2$ and filtered off with suction on a sintered glass plate. The precipitate was washed well with alcohol and ether and sucked dry.

The reduction of $BaCO_3$ to BaC_2 and the subsequent hydrolysis to acetylene were carried out in a 50 cc. Pyrex centrifuge tube closed at the top by a 3-hole rubber stopper (Fig. 1). Glass T-tubes (*A*) were inserted in two of the holes and a separatory funnel (*B*) in the third. Extending through each of the T-tubes was a $\frac{1}{16}$ inch iron electrode (*C*) sealed at the top of the glass tube with

wax. The free ends of the T-tubes served as inlet and outlet for the system. The electrodes extended to the bottom of the test-tube, where a ½ inch piece of No. 36 iron fuse wire was soldered between them.

The $BaCO_3$ was intimately mixed with 200 mg. of fine Mg powder and 25 mg. of SiO_2, put in the test-tube, and covered with a 200 mg. layer of Mg powder. Helium was passed through the system into a trap cooled with liquid air for the condensation of acetylene. A potential of 110 volts applied between the electrodes fired the fuse and started the reaction which required only a few seconds to go to completion. It was essential for the success of this step that the barium carbonate be dry and free from ether. The reaction tube was cooled with a current of compressed air for about 30 seconds. A slow stream of hot water was then introduced from the separatory funnel. The generation of acetylene required only a few minutes.

With this method, a yield of 50 to 70 per cent radioactive acetylene could be obtained in about 10 minutes. This shortened the total time required for the synthesis and purification of the lactate to about 1.5 hours. The over-all yield under favorable circumstances was almost 20 per cent.

The lactate was fed to a fasted rat, the expired CO_2 was collected at half hour intervals, and the liver glycogen was isolated after 2.5 hours. In these steps, the procedure was identical with that previously described (1). The results are given in Tables I and II.

Table I shows that the liver glycogen formed averaged 21 per cent of the administered lactate and contained an average of 3.2 per cent of the administered activity. Comparison of these values with the corresponding figures, 30 and 1.6 per cent, obtained with Type I lactate, indicates that relatively more carbon enters the glycogen from the α or β position than from the carboxyl position of the lactate molecule.

Table II shows that an average of 10 per cent of the administered labeled carbon was excreted as $C^{11}O_2$ in 2.5 hours. This value is considerably lower than the average of 20 per cent found in experiments with Type I lactate. The large errors in some of these figures, particularly those for the last half hours, make it unde-

sirable to attempt to attribute much significance to the absolute values obtained. The results substantiate the view, however,

TABLE I

Radioactivity of Liver Glycogen 2.5 Hours after Feeding C^{11} Lactate, Type II

Experiment No.	Rat weight	Lactate: Fed	Lactate: Absorbed	Liver: Weight	Liver: Glycogen	Glycogen: Formed*	Glycogen: Per cent of lactate fed	Glycogen: Radioactivity: Per cent of amount fed	Glycogen: Radioactivity: Error	Glycogen: Ratio, (9)/(8)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
	gm.	mg.	mg.	gm.	per cent	mg.			per cent	
1	132	115	100	4.55	0.96	38	33	7.1	3	0.21
2†	101	97	50	4.21	0.64	22	23	4.3	4	0.19
3	104	98	64	4.06	0.75	26	26	2.7	3	0.10
4	123	83	79	4.65	0.29	8	10	1.4	14	0.14
5†	151	101	84	4.62	0.61	23	22	3.5	6	0.16
6	137	99	72	4.41	0.31	9	9	0.5	9	0.06
Average							21	3.2		0.14

* Corrected for amount found in livers of fasted controls (0.12 per cent).
† 2 hour experiments.

TABLE II

Excretion of C^{11} in Expired CO_2

Experiment No.	CO_2 expired	Radioactivity as per cent of amount fed: 0-0.5 hr.	0.5-1.0 hr.	1.0-1.5 hrs.	1.5-2.0 hrs.	2.0-2.5 hrs.	Total
	mM						
1	16.8	0.4	1.8	3.2	3.2	4.0	12.6
2*	15.8	1.1	1.9	1.8	5.3		10.1
3	16.5	0.3	1.1	1.3	2.2	1.8	6.7
4	18.4	0.7	1.3	3.0	4.9	4.1	14.0
5*	15.3	0.5	1.1	2.2	2.4		6.2
6	16.8	0.5	1.6	1.7	2.2	0.8	6.8
Average		0.6	1.5	2.2	3.4	2.7	10.0†

* 2 hour experiments.
† 2 hour experiments omitted from the average.

that the fate of the carboxyl carbon atom of lactic acid is quite different from that of the α- and β-carbon atoms.

DISCUSSION

We have previously discussed the mechanism of glycogen formation from lactic acid (1, 4), and proposed a series of reactions which would be consistent with the facts obtained with $C^{11}O_2$ and unlabeled lactate and with $CH_3CHOHC^{11}OOH$. Briefly recapitulated, this hypothesis states that glycogen is formed by a reversal of the steps in the glycolytic process except at the pyruvate → phosphopyruvate stage. Here the following series of reactions is assumed to intervene.

$$
\left.\begin{array}{r}
\text{Pyruvate} + CO_2 \rightarrow \text{oxalacetate} \\
\upharpoonleft\!\downharpoonright \\
\text{malate} \\
\upharpoonleft\!\downharpoonright \\
\text{fumarate} \\
\upharpoonleft\!\downharpoonright \\
\text{succinate}
\end{array}\right\}
\begin{array}{c}
\rightarrow \text{phosphooxalacetate} \\
\downarrow \\
\text{phosphopyruvate} + CO_2
\end{array}
$$

According to this scheme, the original labeled α- or β-carbon atoms of the lactate (Type II) will be converted into glycogen except in so far as the labeled molecules introduced have their specific activity diminished by dilution or interchange reactions in the body. This diminution of specific activity may occur at any of the steps involved. When the carboxyl carbon is labeled as in the Type I lactate experiments, the lactate will be diluted by the same amount; but, after distribution of the C^{11} in both carboxyl groups of the dicarboxylic acids, half of the labeled atoms will be lost in the subsequent decarboxylation. Consequently, the theory predicts that, as a first approximation, the glycogen formed after feeding Type I lactate should contain half as much C^{11} as that formed after feeding Type II lactate. This is the order of magnitude of the difference actually observed in the experimental data.

For a more exact comparison of the data obtained in the experiments with the two types of lactate, some additional corrections should be applied. We have expressed all results in terms of the ratio of the radioactivity found to the glycogen formed. Both numerator and denominator were calculated as per cent of the amount administered. In the case of the Type II lactate, the ratio has been considered a measure of the dilution of the glycogen

precursor in the body. This is true, however, only if no C^{11} enters from another source. Since $C^{11}O_2$ is known to be present, a correction must be applied in each case for the amount of glycogen radioactivity derived from (+4) carbon (4).

The magnitude of this correction can be estimated from the available data by assuming that an average of 11.4 per cent of the carbon atoms has been derived from (+4) carbon, as previously found (4). The amount of glycogen radioactivity so derived then equals

$$0.114 \times \frac{\text{mg. glycogen formed}}{30} \times \frac{C^{11} \text{ excreted as } CO_2}{\text{mM } CO_2 \text{ excreted}}$$

The assumptions underlying this equation have been discussed previously (4).

TABLE III

Comparison of Results with Type I and Type II Lactate

Type I lactate		Type II lactate	
Experiment No.	Ratio	Experiment No.	Ratio
4	0.03	1	0.21
5	0.05	2	0.18
7	0.06	3	0.10
9	0.11	5	0.16
10	0.04		
Average........	0.06		0.16

The correction proved to be insignificant in the case of Type II lactate. For Type I, it amounts to an average of 12 per cent of the total. The final figures selected for comparison of results obtained with the two types of lactic acid (Table III) have all been subjected to this correction.

The ratios listed in Table III have been selected from the two sets of data on Type I and Type II lactate as representing those experiments in which the over-all errors were least. It was felt that only those experiments should be selected in which the calculated percentage conversion of lactate to glycogen did not deviate excessively from the normal average level obtained in the control experiments (1). Experiments in which the glycogen formed amounted to more than 50 per cent or less than 10 per cent of the lactate fed have been excluded as unusual deviations. In the first case, the initial glycogen level of the liver must have been far

above the average control level from which the glycogen increase was calculated, introducing a large error in the latter figure. In the second case, some factor had apparently interfered with the mechanism of glycogen formation. This occasionally happened when the salt content of the administered lactate solution was abnormally high.

The averages of the two sets of corrected ratios in Table III are 0.06 for Type I lactate, and 0.16 for Type II lactate. Although the fluctuations from experiment to experiment are wide, it is believed that the difference observed between the two series is real and consistent with the proposed hypothesis for the formation of liver glycogen from lactate.

We wish to express our appreciation to the Harvard cyclotron group and especially to Dr. B. R. Curtis for their generous co-operation. We also wish to thank the Milton Fund for aid which made this work possible. One of us (A. K. S.) is also indebted to the Ella Sachs Plotz Foundation for a grant-in-aid.

SUMMARY

1. Rats have been fed solutions of lactate containing C^{11} in the α and β positions, and the radioactivity of the expired CO_2 and liver glycogen has been measured.

2. The CO_2 expired in 2.5 hours accounted for about 10 per cent of the administered radioactivity.

3. The liver glycogen formed averaged 21 per cent of the lactate fed and contained 3.2 per cent of the radioactivity.

4. The results are compared with those obtained with lactate labeled in the carboxyl position and are found to be compatible with the previously suggested mechanism of glycogen formation in the liver after lactate feeding.

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GLYCOGEN FORMATION FROM GLUCOSE IN THE PRESENCE OF RADIOACTIVE CARBON DIOXIDE

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The process of glycogen formation in the liver of fasted rats fed lactate has previously been found to involve at least one step in which completely oxidized, or (+4), carbon is incorporated into the 6-carbon glucose chains of the glycogen molecule (1). This incorporation occurs only under circumstances in which glycogen is accumulating. The mechanism of the incorporation is still unknown. There is, however, evidence which indicates that the process may take place by the reaction pyruvate + CO_2 → oxalacetate.

Wood and Werkman (2) have demonstrated the occurrence of this reaction in bacteria. They suggested that the step occurs also in animal tissues. Additional evidence has been provided by other workers (3–7). The data obtained by the use of labeled carbon to study glycogen formation from lactate in rat liver can be explained by the hypothesis that such a carboxylation of pyruvate occurs as a necessary step in the process (8, 9). Other explanations cannot, however, be excluded.

The present paper extends our previous observations to glycogen formation from glucose in the presence of radioactive CO_2. It will be shown that here also (+4) carbon is incorporated into the glycogen molecule.

Methods and Results

Experiments on Rats—The experiments on rats were conducted in a manner identical with that previously used (1). Fasted rats were fed 300 to 600 mg. of glucose in 2 cc. of water, and injected

with $NaHC^{11}O_3$ solutions at half hour intervals. In some cases these solutions were made up without the addition of carrier, so that only a trace of bicarbonate was present. The expired CO_2 was collected each half hour, and, after 2.5 hours, the animal was injected with amytal and the liver removed for glycogen isolation.

The data obtained and the calculated amount of (+4) carbon incorporated into the glycogen are given in Tables I and II. Calculations were made as previously described (1). The results obtained are in no important respect different from those obtained

TABLE I

Radioactivity in Liver Glycogen after $NaHC^{11}O_3$ Administration

Experiment No.	Rat weight	Glucose fed	Solution injected: $NaHC^{11}O_3$ + $NaHCO_3$	Solution injected: NaCl	Solution injected: Total	Liver: Weight	Liver: Glycogen*	Glycogen: Formed†	Glycogen: Radioactivity: Per cent of amount injected	Glycogen: Radioactivity: Per cent error
	gm.	mg.	mM per l.	mM per l.	cc.	gm.	per cent	mg.		
1	98	600	135	9	7.5	4.22	3.10	126	0.84	2.7
3	99	400	99	36	7.5	4.55	2.00	86	1.52	2.9
4	124	400	Trace	146	8.3	4.19	2.34	93	0.99	1.0
5	87	300	"	146	8.3	3.40	0.74	21	0.55	2.7
7	131	400	"	150	8.4	4.99	0.59	24	0.47	1.8
Average								70	0.87	

* Expressed as mg. of glucose per 100 mg. of wet liver.

† Corrected for the amount previously found in the liver of fasted controls (0.12 per cent).

with lactate and $NaHC^{11}O_3$. The total amount of C^{11} excreted as CO_2 averaged 62.7 per cent. Furthermore, C^{11} was found to be present in the glycogen. Calculations showed that an average of 13.1 per cent of the glycogen carbon was derived from (+4) carbon. The fact that this figure is somewhat higher than the average of 11.4 per cent observed with lactate cannot be regarded as significant in view of the large fluctuations in different experiments. That the results in two experiments (Nos. 3 and 5) were higher than 16.6 per cent, likewise does not necessarily mean that more than 1 carbon atom in 6 is involved.

Proof of Presence of C^{11} in Glycogen Molecule—In Experiment 5, an aliquot of the glycogen was hydrolyzed and converted into glucosazone after the addition of a known amount of glucose as a carrier. The osazone isolated represented 6.0 ± 0.3 per cent of the total initial glycogen sample. Its C^{11} content measured 6.2 ± 0.5 per cent of the C^{11} in the total glycogen. This experiment

TABLE II

Proportion of Radioactivity Excreted and Proportion of Glycogen Carbon Derived from (+4) Carbon

Experiment No.	Radioactivity in expired CO_2, per cent of total amount injected						Total CO_2 expired	(+4) carbon incorporated into glycogen	Glycogen carbon derived from (+4) carbon
	0-0.5 hr.	0.5-1.0 hr.	1.0-1.5 hrs	1.5-2.0 hrs	2.0-2.5 hrs	Total			
							mM	*mM*	*per cent*
1	8.3	16.0	14.6	7.8	5.8	52.5	21.2	0.339	8.1
3	14.3	7.3	7.4	12.7	12.7	54.4	17.7	0.195	17.3
4	13.1	14.1	17.1	16.2	12.1	73.7	15.2	0.204	6.6
5	14.3	11.0	12.7	8.4	12.4	58.8	15.6	0.146	20.4
7	13.1	12.6	13.0	17.5	18.0	74.2	17.1	0.108	13.2
Average	....				.....	62.7			13.1

TABLE III

Radioactivity in Muscle Glycogen

Experiment No.	Weight of muscle sample	Glycogen found*	Radioactivity of muscle glycogen		Radioactivity in same amount of liver glycogen
	gm.	*mg*	*per cent*	*per cent error*	*per cent*
6	5.16	15.4	0.049	20.5	0.17
7	5.21	15.8	0.022	6.6	0.25

* Expressed as mg. of glucose.

shows that all or nearly all (at least 90 per cent) of the C^{11} measured in the glycogen is a part of the glucose carbon chain.

Radioactivity of Muscle Glycogen—Muscle glycogen was isolated for radioactivity determination in two cases. The results are given in Table III. Appreciable quantities of C^{11} were found, though the amount present per mg. of glycogen was not as great as that found in the liver. Comparison of these values with those

previously obtained after lactate feeding shows that more C^{11} appears in muscle glycogen after glucose feeding. This is consistent with the fact that glycogen is known to be deposited in the muscles after administration of glucose to fasted rats (10). Only the newly formed glycogen would be expected to contain labeled carbon.

Experiments with Rabbit Liver Slices—The preceding results on rats indicated that the glycogen deposited in the liver after glucose feeding incorporated as much (+4) carbon as that deposited after lactate feeding. To test whether the steps involved in the synthesis of glycogen from glucose really necessitated the incorporation of (+4) carbon, a system was required in which the formation of glycogen from precursors of lower molecular weight does not occur. Ostern, Herbert, and Holmes (11) have shown that liver slices of fasted rabbits will synthesize glycogen in a suitable inorganic medium containing high concentrations (1 per cent) of glucose. Under the same conditions, glycogen formation from lactate, pyruvate, succinate, or fumarate occurs only to a small extent or not at all. This system was, therefore, chosen for investigation.

Preliminary experiments were run in order to determine the conditions necessary for a maximum yield of glycogen. Since the conditions chosen differed somewhat from those employed by Ostern *et al.*, the procedure used in one experiment with C^{11} will be described in detail.[1]

4 cc. of a solution containing 0.09 M KCl and 0.033 M $KHCO_3$ per liter were added to 4 cc. of a solution of the following composition: 0.020 M $Mg(HCO_3)_2$, 0.070 M KCl. To this mixture were added 80 mg. of glucose and liver slices from a rabbit fasted 24 hours. The gas space of the vessel employed was filled with 95 per cent O_2, 5 per cent CO_2. The vessel was stoppered and shaken at 37° for 1 hour. After the addition of 4 cc. of 80 per cent KOH, the glycogen was isolated by the usual procedure. Chemical determination of the amount of glycogen present was made on an aliquot. A separate determination of the glycogen content of the liver slices was made at the beginning of the incubation period. The results obtained in this experiment and in another similar one

[1] A study of the rôle of ions in the *in vitro* formation of glycogen will be published separately.

employing somewhat larger quantities of medium and tissue are given in Table IV.

The glycogen formation in the two experiments amounted to 9.6 and 18.8 mg. respectively. This glycogen contained 0.17 and 0.45 per cent of the total C^{11} present. A control experiment showed the C^{11} was not present as a contaminant. In order to assess the significance of these results, it is necessary to calculate what proportion of the carbon atoms of the glycogen are represented by the C^{11} which it contains. Such a calculation can only be a rough approximation, since it is impossible to ascertain with accuracy the specific activity of the (+4) carbon involved. At the beginning of the experiment, all the C^{11} may be assumed to be evenly distributed in the (+4) carbon of the medium.

TABLE IV

C^{11} in Glycogen Formed from Glucose in Rabbit Liver Slices

Experiment No.	1	2
Amount of tissue, *gm.*	1.86	3.22
Initial glycogen, %	0.26	0.26
Final glycogen, %	0.77	0.85
Glycogen formed, *mg.*	9.6	18.8
C^{11} in glycogen, as % total C^{11} present	0.170 ± 0.004	0.450 ± 0.005

The amount of (+4) carbon was 0.29 mM in the first experiment and 0.40 mM in the second. If this specific activity was retained throughout the experiment, the C^{11} found in the glycogen would represent 0.0005 mM of carbon or 0.16 per cent of the glycogen carbon in the first experiment, and 0.0018 mM of carbon or 0.29 per cent of the glycogen carbon in the second experiment. These figures represent minimum values, since the amount of (+4) carbon represented by a given amount of C^{11} increases throughout the experiment as more CO_2 is produced by the tissue and as the C^{11} becomes distributed in the gaseous phase. The difference in intracellular and extracellular specific activity may also be significant. If this last factor is neglected, a minimum value can be assigned to the specific activity by assuming that the C^{11} is evenly distributed in the total amount of (+4) carbon present

in liquid and gas at the termination of the experiment.[2] This was estimated to be 0.77 mM in Experiment 1, and 1.30 mM in Experiment 2. If these concentrations existed throughout the experiment, the C^{11} found in the glycogen would represent 0.0013 mM of carbon or 0.41 per cent of the glycogen carbon in Experiment 1, and 0.0059 mM of carbon or 0.94 per cent of the glycogen carbon in Experiment 2. The largest value, about 1 per cent, is well below the value of 13 per cent observed when glycogen was formed from glucose *in vivo*. Furthermore, the minimum specific activity calculated above would have to be decreased by a factor of 16 if 1 carbon atom in each glucose unit were involved. Though the possibility of such a difference between the approximated and the real conditions cannot be denied, it is unlikely. The (+4) carbon incorporated is probably to be ascribed to the small but definite amount of glycogen usually formed when the liver slices are incubated under the same conditions in the absence of added glucose.

DISCUSSION

Although a definite amount of (+4) carbon was incorporated into the glycogen formed from glucose in rabbit liver slices, the amount involved seems too small to indicate that the process involving the incorporation is a necessary step in the glucose to glycogen conversion. This conclusion is consistent with the findings of Cori and his coworkers (12, 13) that glucose can be enzymically converted into glycogen *in vitro* by the following direct steps.

Glucose → hexose-6-phosphate → hexose-1-phosphate → glycogen

The fact that 13 per cent of the glycogen formed *in vivo* after glucose feeding is derived from (+4) carbon may mean either that an entirely different process is here involved, or that the glucose is broken down to a 3-carbon unit before being resynthesized to glycogen by a series of reactions similar to those which occur after lactate feeding. However, it seems most likely that the existence of equilibria between the various chemical components involved in the carbohydrate system causes a distribution of labeled carbon

[2] The possibility of loss of C^{11} by its entrance into organic molecules in any appreciable amount is also neglected.

atoms introduced at any one stage into all the molecular species involved. Such a distribution could be expected even though there were no net formation of glycogen from 3- or 4-carbon precursors. The apparent dilution of labeled lactate fed to the rat lends support to such a concept, which has already been shown by Schoenheimer and Rittenberg and their coworkers (14) to be applicable to many biological reactions in the intact animal.

From the observations made in this and the preceding papers of this series, the hypothesis is proposed that though different chemical substances may give rise to an increase in liver glycogen, they may be regarded as interchangeable if they are capable of being brought into enzymic equilibrium in the body with the key precursor, pyruvic acid, which in turn condenses with CO_2 to provide again the essential 4-carbon acids.

We wish to express our appreciation for their generous cooperation to the members of the Harvard cyclotron group and especially to Dr. B. R. Curtis. We also thank the Milton Fund for aid which made this work possible.

SUMMARY

1. Radioactive bicarbonate was injected and glucose was fed to fasted rats. The liver glycogen formed incorporated an amount of C^{11} corresponding to 13 per cent of the carbon atoms of the glycogen.

2. A small amount of C^{11} was also found in the muscle glycogen.

3. The formation of glycogen from glucose in rabbit liver slices in the presence of labeled (+4) carbon also resulted in the appearance of some C^{11} in the glycogen molecule. Here, however, the amount of (+4) carbon incorporated was considerably less than *in vivo*.

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THE UTILIZATION OF CYSTEINE AND CYSTINE BY RAT LIVER WITH THE PRODUCTION OF HYDROGEN SULFIDE*

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In the course of work on the synthesis of vitamin C by animal tissues (1) we have had occasion to add cysteine to preparations of rat liver. In every case (with liver slices, homogenized liver, and liver extracts) a production of H_2S was observed. Although it is well established that certain bacteria contain an enzyme system capable of producing H_2S from cysteine, the only report we could find in the literature indicating that mammalian tissue contains such an enzyme system was that of Fromageot, Wookey, and Chaix (2). These authors reported the presence of such an enzyme in dog liver. The present work was undertaken to study the conditions necessary for the production of H_2S by preparations from the liver of the rat and a few other species and to study the other products that are formed from cysteine and cystine. During the course of the work Laskowski and Fromageot (3) have extended their observations to some other species and have described certain properties of the enzyme preparation from dog liver.

EXPERIMENTAL

The H_2S produced could be determined quantitatively in the usual Warburg vessels by absorbing it in cadmium acetate placed in the center inset and treating the yellow solution and precipitate with excess iodine and acid at the end of the experiment. The

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A preliminary account of these experiments was presented before the Thirty-fifth annual meeting of the American Society of Biological Chemists at Chicago, April 16, 1941 (*J. Biol. Chem.*, 140, p. cxxiii (1941)).

acid dissolved the sulfide and the iodine oxidized it to free sulfur. The remaining iodine could be titrated in the usual way. Controls run by adding sodium sulfide from the side arm of the vessels indicated that it could be recovered within 5 per cent as long as the solutions contained nothing that reacted with the H_2S. This method has been used for all of the present work that was done in Warburg vessels. When larger amounts of material were used, a similar absorbing inset was arranged or the H_2S was aerated into cadmium acetate.

The first experiments were all carried out with tissue slices suspended in either phosphate or bicarbonate buffer. The production of H_2S was greater under anaerobic than under aerobic conditions, so that in the experiments that follow the conditions were always anaerobic unless stated to be otherwise.

Rat, dog, human, beef, rabbit, pork, and guinea pig livers were tested. They all produced some H_2S from cysteine. Rat liver was the most active. It produced on an average, 0.3 mg. of sulfur as H_2S per gm. of fresh liver in 2 hours when cysteine was present in excess. If the activity of rat liver is taken as 100, the activities of the others were 60, 50, 18, 5, 3, and 1 respectively. The activity of rat kidney was 1, that of rat muscle was less than 1, and that of brain was 0. In the rest of this report only the results with rat liver will be considered. The rats had been receiving a Purina dog chow diet *ad libitum*.

Essentially all of the activity of liver could be extracted with 0.9 per cent NaCl after grinding in a mortar with sand. 2.0 ml. of saline per gm. of liver were usually employed. After centrifugation this yielded about 2.0 ml. of extract. Some additional active material could be obtained by a second extraction, but the concentration was much lower. Such saline extracts were relatively stable but lost activity slowly even at 0°, becoming almost completely inactive in 10 days. Such extracts could be dialyzed in cellophane tubes against cold running tap water for 24 hours without appreciable loss in activity. A heavy precipitate developed as a result of such dialysis, but it did not contain appreciable amounts of active material. The supernatant from this precipitate was more stable than the original extract. Heating at 85° for 10 minutes inactivated all the extracts tested. Inactivation was slow at 50°, but it was rapid at 60°.

The extracts were most active at pH 7.4 to 7.8. The active material could be precipitated by saturating the solution with ammonium sulfate or by the addition of organic solvents such as alcohol or acetone. Toluene or chloroform could be employed as antiseptics without effect on the activity. 0.007 M KCN caused almost complete inhibition of activity as judged by the H_2S obtained. 0.03 M NaF had no inhibiting effect.

Liver slices or extracts produced small amounts of H_2S without added substrate. Controls were run to determine this amount but in many cases it was negligible in 2 to 3 hour experiments compared with the amounts produced from added cysteine. The addition of glutathione instead of cysteine resulted in the production of only very small amounts of H_2S. These small amounts can probably be accounted for by a small production of cysteine from the glutathione. Concentrations of methionine and thioglycolic acid similar to that of cysteine did not yield significant quantities of H_2S within 2 to 3 hours. *dl*-Homocysteine yielded small amounts of H_2S but the enzyme activity was only about one-tenth as great with this substrate as with cysteine.

The amounts of H_2S produced from varying amounts of added cysteine and enzyme preparations in different times are shown in Table I. It may be observed that in a number of cases the H_2S produced was approximately equivalent to 50 per cent of the cysteine added. The same approximation holds for many experiments not shown in Table I. However, it is apparent from the 5 hour experiments shown and from Table II that 50 per cent is not to be regarded as an end-point.

A comparison of the cysteine consumed and the H_2S produced is shown in Table II. In order to distinguish between cysteine that may have been oxidized to cystine and cysteine that was converted to other material we have determined both the reducing power to iodine and the cystine content after the cysteine was oxidized by air. The titration with iodine was carried out after the solution had been deproteinized with metaphosphoric acid or trichloroacetic acid and made 1.0 M with sulfuric acid. The results were compared with titrations of the same enzyme preparation plus known amounts of cysteine. The Sullivan and Hess method (4) for cystine was applied to the neutralized deproteinized solution after a trace of copper sulfate had been added to serve as

a catalyst and the solutions aerated until the nitroprusside reaction was negative. The colors were compared with controls made from known amounts of cystine or of cysteine treated in the same way. The difference between the results for cystine and the iodine titration is thought to indicate a production of cystine from cysteine during the experiment. The experiments were carried out in

TABLE I

Hydrogen Sulfide Production by Liver Extract Plus Cysteine

Phosphate buffer, pH 7.6 under nitrogen; T, 37°.

Enzyme used	Time of reaction	Cysteine added	H_2S produced
ml. saline extract	*hrs.*	*mg. S*	*mg. S*
2.0	2.0	0.18	0.08
2.0	2.0	0.35	0.19
2.0	2.0	0.53	0.25
2.0	2.0	0.70	0.32
2.0	2.0	1.05	0.39
2.0	2.0	1.40	0.38
0.5	2.0	0.70	0.03
1.0	2.0	0.70	0.11
1.5	2.0	0.70	0.17
2.0	2.0	0.70	0.21
2.5	2.0	0.70	0.25
2.0	1.0	0.70	0.15
2.0	2.0	0.70	0.31
2.0	3.0	0.70	0.36
2.0	5.0	0.70	0.42
2.0	5.0	0.70 + 0.70*	0.63
2.0 + 2.0†	5.0	0.70	0.40

* After 3 hours, the second 0.70 mg. portion of cysteine was added (causing an increased H_2S production).

† After 3 hours, the second 2.0 ml. of extract were added (without an increased H_2S production).

phosphate buffer under N_2. The evidence that the nitrogen did not contain enough oxygen to produce such an amount of cystine by simple autoxidation is that if the enzyme preparation was omitted the cystine was not found. The formation of the cystine may, however, be entirely independent of the reaction producing H_2S. It is apparent from Table II that the H_2S obtained does not

account for all the sulfur that disappeared as cysteine or cystine. This point will be discussed later. In ten experiments not shown in Table II the H_2S obtained accounted for an average of 66.6 per cent of the sulfur of the cysteine consumed.

The above results bring up the question of what happens to the rest of the cysteine molecule. If we assume that no preliminary reactions are involved, there would seem to be three simple reactions by which H_2S might be formed from cysteine. They are

TABLE II

Cysteine Lost and Hydrogen Sulfide Formed

2.0 ml. portions of liver extract in phosphate buffer, pH 7.6, under nitrogen; T, 37°. The first six experiments were with the same enzyme preparation; the last four experiments were each with a different enzyme preparation.

Cysteine added	Time of reaction	Cysteine remaining (I_2 titration)	Cysteine + cystine remaining	H_2S produced	Cysteine* S loss recovered as H_2S
mg S	*hrs*	*mg S*	*mg S*	*mg. S*	*per cent*
0.70	1	0.42	0.16	0.15	62.5
0.70	2	0.18	0.34	0.31	86.0
0.70	3	0.07	0.19	0.36	70.5
0.70	5	0.03	0.17	0.42	79.2
0.70	5	0.06	0.17	0.40	75.5
1.40	5	0.10	0.48	0.63	68.5
0.70	2	0.45	0.53	0.09	52.9
0.70	2	0.39	0.47	0.13	56.5
0.70	2	0.25	0.39	0.25	80.5
0.70	2	0.18	0.21	0.31	63 2
Average					69.5

* Cysteine S here includes the cystine S, since the latter represents available S, essentially unchanged by the enzyme. The cysteine S lost is given by the first column minus the fourth column.

(1) $CH_2SHCHNH_2COOH + H_2O = CH_2OHCHNH_2COOH + H_2S$

(2) $CH_2SHCHNH_2COOH + H_2 = CH_3CHNH_2COOH + H_2S$

(3) $CH_2SHCHNH_2COOH = CH_2{=}CNH_2COOH + H_2S$

The possible occurrence of Reaction 1 was tested by two methods of detecting serine. One was the method of Rappoport (5), which consists of deaminizing and treating the resulting glyceric acid

with naphthoresorcinol to give a blue color. The application of this method to liver extract gave very strong brown colors. The brown color could be diminished by using a dialyzed enzyme preparation, removing the products of the reaction by dialysis, and applying the test to the concentrated dialysate. Added serine gave an easily detected color under these conditions, but we could not obtain any blue color from the test solutions. The other method consists of treating the solutions with ninhydrin, removing the volatile aldehydes, and forming the dimedon compound of the remaining aldehydes (6). No glycolaldehyde compound could be obtained. From these results we conclude that Reaction 1 did not occur to an appreciable extent.

The possible occurrence of Reaction 2 was tested by two methods of detecting alanine. One was the method of Virtanen *et al.* (7) which consists of reacting with ninhydrin and determining the acetaldehyde formed by aerating it into bisulfite as in a well known lactic acid determination. Any preformed volatile aldehydes were first removed. The other method was that of Block *et al.* (8) which consists of deamination, oxidation with lead tetraacetate, and aeration of the acetaldehyde into *p*-hydroxydiphenyl. The difference in the red color produced before and after deamination represents the alanine. Both of these methods gave consistently positive results for the presence of alanine in the solutions. Quantitatively, however, the results varied all the way from an amount of alanine equivalent to about 1 per cent of the H_2S produced to about 26 per cent of the H_2S produced. In no case could the alanine be considered the main product of the reaction and the variability of the amount produced suggested that it was either a secondary product (*i.e.*, produced from the primary products) or the main product of a side reaction.

The product of Reaction 3, aminoacrylic acid, is an unstable compound (9) and its fate if produced in an extract is not certain. It is an isomer of the imino acid (dehydroalanine) believed to be produced in the enzymatic oxidation of alanine. The imino acid is believed to break down spontaneously to form pyruvic acid and ammonia; therefore it seemed probable that aminoacrylic acid might do the same. However, tests for these two products when either liver slices or certain liver extracts were employed showed only small amounts of either to be present. Fromageot, Wookey,

and Chaix (2) reported that ammonia was not formed in their experiments. However, since both pyruvic acid and ammonia can be utilized by liver, it seemed likely that a somewhat purified enzyme preparation might make their demonstration easier if they were formed. The simplest way found of preparing an extract sufficiently purified for this purpose was to treat the saline extract with an equal volume of chloroform (10) and shake vigorously for 20 minutes. After centrifugation the aqueous layer could be decanted. The volume recovered was somewhat diminished (about 80 per cent), but the activity per unit volume was practically unchanged. At the end of 2 hour experiments with such extracts, the presence of both pyruvic acid and ammonia was readily demonstrated. The amounts found are recorded in Table III. The ammonia was determined in the deproteinized solutions by aerating it from an alkaline solution into dilute HCl followed by nesslerization. Controls were made up in the same way, but stopped at zero time. The pyruvic acid was determined on the unfractionated solutions by lowering the pH of the solutions to 5.0 with acetate buffer and measuring the CO_2 produced by a carboxylase preparation from yeast (11).

The use of this method requires some explanation, for it frequently gives low recoveries of pyruvic acid added to liver extracts. Different extracts prepared as described above differed considerably in this respect. With some of them, almost theoretical recovery was obtained, while with others only low recoveries were obtained even on very short standing. For example, in one case 429 c.mm. of CO_2 were obtained from water and only 317 c.mm. from an extract after standing 10 minutes at pH 5.0 in the absence of oxygen. In another case 211 c.mm. were obtained from water and only 77 c.mm. from an extract. In general the recovery was lower the longer the pyruvate was in contact with the extract. The addition of arsenous acid to the extracts improved the recovery of added pyruvic acid, but did not prevent some loss. The results suggest that some pyruvate-enzyme system was active to a varying degree in our preparations. A factor could be used to correct for the low recovery as far as added pyruvic acid is concerned, but in the present experiments any correction would be rather uncertain, for the pyruvic acid was produced gradually over a considerable period of time. We have accordingly not applied

any correction, even though we have shown the recovery to be low in the extracts used. The figures in Table III are those actually determined.

In three experiments the contents of seven or more Warburg vessels were pooled and the pyruvic acid was isolated as the 2,4-dinitrophenylhydrazone. The isolation was accomplished by

TABLE III

Conversion of Cysteine to Hydrogen Sulfide, Pyruvic Acid, and Ammonia

2.0 ml. of chloroform-treated liver extract in phosphate buffer, pH 7.6; under nitrogen; T, 37°; time of reaction, 2 hours; cysteine added, 20.0×10^{-6} mole.

H_2S produced	Pyruvic acid produced	Ammonia produced
mole $\times 10^{-6}$	*mole* $\times 10^{-6}$	*mole* $\times 10^{-6}$
8.6	5.2	9.5
11.0	4.9	10.1
8.0	5.9	8.9
9.7	4.4	6.0
9.1	7.6	7.6
9.7	4.9	5.3
4.1	2.3	2.4
7.2	4.7	4.1
6.7	6.5	
7.5	4.0	
8.4	5.1	
7.0	3.7	
9.7	5.0	
5.5		7.3
11.4		15.1
3.6		3.5
Average....8.0	4.9	7.3
" 2.6*	4.4	9.3

* Average of six aerobic experiments.

extraction with ethyl acetate and then with sodium carbonate (12, 13). Each extraction was repeated three times. The final precipitation was made by acidifying a sodium carbonate solution. The precipitate was washed with 0.1 M HCl and dried *in vacuo* over drierite. The average yield was 52.5 per cent of the carboxylase value. The melting point was 214° (uncorrected). The mixed melting point with known pyruvic acid dinitrophenylhydrazone

was 214°. An equal amount of extract without added cysteine did not yield any product by the same method.

From the above results it seems clear that pyruvic acid and ammonia are major products in the utilization of cysteine under the conditions of study. The yields indicate that other products were probably present. The isolation and identification of these other products were attempted, but not concluded. It appears likely that they are, at least to a considerable extent, formed by secondary reactions involving pyruvic acid, ammonia, H_2S, and possibly cysteine. The following points are in harmony with this view. Pyruvic acid is known to react with liver preparations (14–20), with ammonia in the presence of tissue preparations (14–16, 18, 19), with amino acids (20, 21), with H_2S,[1] and with the —SH group of cysteine (22). The H_2S obtained is always less than the cysteine or cystine used. By repeated additions of cysteine to the same enzyme preparation, the amount of H_2S produced could be increased far beyond the amounts reported. The enzyme was still active after 24 hours. However, at the end of such 24 hour experiments very little or no pyruvic acid could be demonstrated. Since the pyruvic acid could be demonstrated in relatively good yields in short experiments, it seems clear that it must have been converted to other products.

While the formation of pyruvic acid, ammonia, and H_2S is compatible with the mechanism indicated in Reaction 3, there are other equally simple alternatives. For example,

$$CH_2SHCHNH_2COOH = CH_2SHC{=}NHCOOH + H_2 \tag{4}$$
$$CH_2SHC{=}NHCOOH + H_2O = CH_2SHCOCOOH + NH_3 \tag{5}$$
$$CH_2SHCOCOOH + H_2 = CH_3COCOOH + H_2S \tag{6}$$

Such considerations have an obvious bearing on the question of naming the enzyme involved. Fromageot and coworkers have suggested the name desulfurase. Since the reaction is relatively specific for cysteine, such a name should at least be qualified and made cysteine desulfurase. However, in the light of the possibility shown in Reactions 4, 5, and 6 this name may not be applicable. Perhaps, the choice of name should await the elucidation of the mechanism.

When cystine was added to the liver preparations instead of

[1] Unpublished results of this laboratory.

cysteine, smaller amounts of H_2S were obtained. However, when the solutions were analyzed for ammonia and pyruvic acid, these products were found in amounts essentially the same as when cysteine was used. The amounts obtained are recorded in Table IV along with other pertinent data.

The pyruvic acid was isolated from the cystine experiments as the dinitrophenylhydrazone in the same way as described above for the cysteine experiments. It melted at 214°.

TABLE IV

Reactions of Cystine Induced by Liver Extract

The production of various products by 2.0 ml. of chloroform-treated liver extract in phosphate buffer, pH 7.6; under nitrogen; T, 37°; cystine added, 20.0×10^{-6} mole.

H_2S produced	S produced	Pyruvic acid produced	Ammonia produced	Cysteine produced	Cystine lost (exclusive of cysteine formed)
mole $\times$ *10*$^{-6}$	*mole* $\times$ *10*$^{-6}$	*mole* $\times$ *10*$^{-6}$	*mole* $\times$ *10*$^{-6}$	*mole* $\times$ *10*$^{-6}$	*mole* $\times$ *10*$^{-6}$
4.1	5.6	3.4	6.1	2.4	7.3
3.0	8.9	3.3	4.1	6.0	10.5
5.7*		4.8	6.1	0.4	6.5
2.1*	10.0	6.4	5.3	6.0	10.7
3.3		4.2	8.8		
2.1	3.4	4.0	7.0	12.0	
4.0	10.3	7.1			
4.4	6.6	6.5		8.6	
5.0	7.5	5.4			
3.0	2.0	2.4			

* Aerobic experiments.

The relatively low H_2S values obtained with cystine suggested the following considerations. If H_2S were produced in a solution containing cystine, it seemed possible that an interaction would occur as follows: $RSSR + H_2S = 2RSH + S$. That cysteine was formed in the reaction was easily demonstrated by iodine titration and the values found are shown in Table IV. That sulfur was present was demonstrated by pooling the contents of several vessels, adding to the solution an equal volume of concentrated HCl, centrifuging, washing the precipitate with concentrated HCl, extracting the residue with absolute alcohol, evaporating the alcohol, oxidizing with $HNO_3 + KClO_3$, and

precipitating the sulfate with $BaCl_2$. Since such a method is not readily applied on a micro scale, we have sought another one and found that the reaction of sulfur with sulfhydryl groups could be made the basis for an adequate method. Guthrie (23) has proposed a method of determining glutathione based on the measurement of the H_2S produced by the reaction of glutathione with excess sulfur. The reaction involved is $2GSH + S = GSSG + H_2S$. As Guthrie realized, the reaction is not specific for glutathione, but is given by other sulfhydryl groups. We have tested the reaction, using both cysteine and thioglycolic acid, and have found it to be satisfactory. To determine S by such a method it is only necessary to use an excess of the sulfhydryl compound and to measure the H_2S produced. We have carried out the reaction in Warburg vessels, absorbing the H_2S in cadmium acetate placed in the inset as described above. Quantities of sulfur from 0.05 to 1.0 mg. could be determined with an accuracy of ±5.0 per cent by using 1.0 ml. of 0.25 N cysteine and allowing the reaction to proceed for 2 hours at 37° and pH 6.8. The sulfur to be determined was dissolved in absolute alcohol after most of the other material present had been dissolved in concentrated HCl. 1.0 ml. of hot alcohol readily dissolves 0.5 mg. of S and the solution remains clear on cooling. 2 ml. of such a solution can be added to a Warburg vessel containing an aqueous buffer and cysteine solution in such concentration that the total volume is 3.0 to 3.5 ml. Some precipitation may occur but this does not prevent the reaction from taking place. The reaction will occur in the absence of any solvent other than water, but it is considerably slower under such conditions. Some results obtained by this method are shown in Table IV. Small amounts of sulfur were also obtained in several cysteine experiments.

Aerobic experiments were carried out similarly with both cysteine and cystine. The formation of pyruvic acid and ammonia from each of them and the formation of sulfur from cystine occurred just as readily as anaerobically (Tables III and IV). The amounts of H_2S obtained from cysteine were considerably smaller (Table III), which may indicate the formation of sulfur and of sulfate (24).

Serine and threonine were tested for keto acid formation under the same conditions as described above. With chloroform-treated

extracts that showed good pyruvic acid formation from cysteine and cystine no carboxylase reaction was obtained from either of the hydroxyamino acids under either aerobic or anaerobic conditions.

DISCUSSION

The extent to which the reactions described occur physiologically is not known. Presumably the formation of H_2S would be limited by the relatively low concentrations of free cysteine and cystine that occur normally. When cysteine or cystine is fed or injected, however, the reactions reported here would be expected to play a significant rôle. The phlorhizinized dog is known to convert the carbon of ingested cysteine to glycogen (25). This might well occur via the formation of pyruvic acid, as reported here. The sulfur of ingested cysteine is known to be converted in part, at least, into inorganic sulfate by the intact animal (25) and by liver slices (24). The sulfur of ingested cystine is largely converted to sulfate by an intact animal (26) but not by tissue slices (24). A common assumption has been that the oxidation of cysteine sulfur occurs before it is split from the carbon. This procedure is, of course, possible, but the oxidation may equally well occur after the sulfur is converted to H_2S, as has been demonstrated by the injection of Na_2S (27) intravenously and as probably occurs with the H_2S absorbed from the intestinal tract. H_2S is, of course, a very toxic substance (28). The effect of its production in the liver would undoubtedly depend upon the relative rates of its production and detoxication. It is now well recognized that the feeding of free cysteine or cystine may be very toxic to young white rats (29, 30). The fact that this toxicity may be prevented by labile methyl groups (31–33) does not necessarily explain why the cysteine is toxic. One reason for the toxicity may be the production of H_2S. The fact that dimethyl sulfone has been isolated from tissues (34, 35) may indicate that H_2S is in part detoxified by methylation. If the production of H_2S were a principal reason for the toxicity of cysteine, one might expect that the toxicity would not necessarily be proportional to the amount of cysteine or cystine fed but only to the amount converted to H_2S; *i.e.*, the toxicity would be determined by the amount and activity of the enzyme present. On this basis cysteine should not be toxic to

guinea pigs. It is interesting to note that in a recent paper Griffith (32) reported that the damage done by feeding cystine to rats is not proportional to the amount of cystine fed and that the amount of choline required to protect against 1 per cent cystine is no greater than the amount required to protect against 0.3 per cent cystine.

Although the reversal of the reaction described here has not yet been demonstrated, it may be noted that the combination of H_2S with aminoacrylic acid has been postulated (9) as a mechanism for the formation of cysteine in tissues. An approach to the reaction has been described recently (36) in which Na_2S is added to proteins in alkaline solution. The alkaline proteins are thought to contain peptides of aminoacrylic acid formed by dehydration of serine molecules and so the addition of Na_2S results in cysteine formation. A reversal of the H_2S-forming reaction may be involved in the conversion of methionine to cysteine.

It should also be pointed out that sodium hydroxide in the inset of a Warburg vessel absorbs H_2S produced in the vessel. Consequently, experiments on respiratory quotients of liver preparations in the presence of cysteine, performed by using two vessels one of which contains alkali, may be seriously in error, for the contents of these two vessels are subjected to different H_2S concentrations.

SUMMARY

The production of hydrogen sulfide from cysteine and from cystine by the liver of the rat and certain other species has been described. Pyruvic acid and ammonia have been demonstrated to be other principal products of the reaction on both substrates. Elementary sulfur has been shown to be formed from cystine and to a small extent from cysteine. A method of determining small amounts of sulfur has been described. Some properties of the enzyme system involved have been studied and the possible importance of the reactions has been discussed.

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STUDIES IN NICOTINIC ACID METABOLISM

III. METABOLISM AND SYNTHESIS OF NICOTINIC ACID IN THE RAT*

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The rat is a mammalian species of laboratory animal in which the synthesis of nicotinic acid has been definitely demonstrated. The literature is reviewed by Shourie and Swaminathan (1) and by Dann and Kohn (2). Shourie and Swaminathan's (1) conclusion that rats synthesize nicotinic acid is based on their finding an excess of nicotinic acid in the urine and feces of rats fed over a long period of time a diet very low in nicotinic acid. Since they did not determine the trigonelline in the excreta, and since trigonelline, as will be seen below, constitutes a major part of the normal nicotinic acid excretion in rats, their conclusions are based on an underestimate of the capacity of the rat to synthesize nicotinic acid. The analyses of the tissues which these authors quote also show approximately the same nicotinic acid content in the liver, muscle, and blood of rats fed on low and high intakes of nicotinic acid.

Dann and Kohn (2) showed, by the coenzyme estimations in tissues, that rats are able to synthesize coenzyme and nicotinic acid itself on low nicotinic acid intake in their diets. In the later work of Dann (3) the above conclusions were definitely confirmed by direct analysis of nicotinic acid in tissues of rats raised on diets low in nicotinic acid.

In view of the facts that the rat, in distinction to man and dog, synthesizes nicotinic acid and, therefore, does not require it from

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external sources, it was deemed particularly interesting tostudy the metabolism of nicotinic acid and its derivatives in this species. As stated above, the only metabolic study reported thus far in the literature, that of Shourie and Swaminathan (1), was inadequate, because it did not include the determination of trigonelline in the excreta.

The data presented in this communication deal with the following topics: the relation of nicotinic acid excretion to food intake and the fate of nicotinic acid and its derivatives when administered to rats; the synthesis of nicotinic acid in rats from known nitrogenous precursors; and saturation experiments.

Urinary Excretion of Nicotinic Acid in Rats on Diets Containing Protein and Metabolism of Ingested Nicotinic Acid Derivatives

Adult white rats of 250 to 300 gm. were used in this study. They were placed in groups of three or four rats of the same sex in roomy cages permitting the separate collection of urine and feces. The excreta were analyzed by the methods described previously (4, 5).

In the first series of experiments three groups, one of females and two of males, of four rats each were placed on a diet consisting of crude casein 14 gm., yellow corn-meal 76 gm., cottonseed oil 5 gm., cod liver oil 2 gm., $CaCO_3$ 1 gm., $CaHPO_4$ 1 gm., and NaCl 1 gm. The corn-meal and the salts were cooked in a double boiler with 400 cc. of water; the mixture was allowed to cool somewhat and the casein and the oils were then thoroughly mixed in. A supplement sufficient to provide 15 γ of thiamine and 10 γ of riboflavin per rat per day was also incorporated into the mixture. This is substantially the same diet as that used by Sarett (6) in Paper II of this series dealing with the metabolism of nicotinic acid in dogs.

The diet as fed contained 1.2 γ of nicotinic acid per gm. Its trigonelline content was too low to be determined accurately. The rats consumed on the average 110 gm. per animal per day. Water was allowed *ad libitum* in addition. Adequate urine volumes of 30 to 50 cc. per rat per 24 hours were usually obtained. The rats maintained their weight on this diet, gaining on the average about 15 gm. in 30 days.

On an average intake of about 110 gm. of the above diet, the daily intake per rat was 5 gm. of protein (1.5 gm. of casein + 3.5

gm. of corn-meal protein) and 130 γ of nicotinic acid. The three groups of rats were maintained for 6 days on this diet, then the intake of food was reduced by one-half for 4 days, and finally the rats were fasted for 5 days. The data in Table I show the averaged results for twelve rats of the analyses of 24 hour urines collected under these dietary conditions.

TABLE I

Urinary Excretion of Nicotinic Acid in Rats As Affected by Food Intake and Ingestion of Nicotinic Acid and Its Derivatives

The values given are in micrograms per rat per day, the averages for twelve adult rats.

Day	NA*	Trigonelline	Total†	Diet
1	41	220	239	Corn-meal + casein, 6 days; protein intake, 5 gm. per rat per day
3	45	193	219	
6	28	102	120	
10	26	86	103	After 4 days on one-half of above diet
15	21	77	90	After 5 days fasting
1	26	561	535	Above diet, after single dose of 1.5 mg. trigonelline
2	26	155	166	
3	26	122	136	
1	654	301	925	Above diet, after single dose of 2.2 mg. nicotinuric acid (1.5 mg. nicotinic acid)
2	45	149	178	
1	101	551	624	Same diet, single dose of 1.5 mg. nicotinamide
2	32	152	168	
4	244	957	1106	Same diet, single dose of 3.0 mg. nicotinic acid
5	37	148	170	
7	197	858	969	3.0 mg. nicotinic acid + 0.5 gm. glycine

* NA includes all nicotinic acid derivatives hydrolyzable by boiling with 6 N HCl.

† Total represents the sum of NA and trigonelline calculated in terms of nicotinic acid.

In the first 6 days of ingesting this diet the NA fraction (total hydrolyzed with 6 N HCl) decreased from about 45 γ per rat per day to 28 γ. The trigonelline fraction which was initially 220 γ per rat per day declined to 102 γ. These decreases may be explained by the fact that, before being placed on the experimental diet, the rats had been maintained for a long time on a ration con-

taining a liver concentrate and a commerical biscuit including a large proportion of bean meal of high trigonelline content.

The average output per rat per day of the total nicotinic acid derivatives amounted to 120 γ. This corresponds to the estimated average intake of 130 γ of nicotinic acid per day in the food. Unfortunately, the nicotinic acid content of the feces was not determined in this experiment. From subsequent experiments (Tables IV and V) it was found that the total nicotinic acid content of the feces varied between 40 and 90 γ per rat per day. It is apparent, therefore, that on an intake of approximately 0.4 mg. per kilo of body weight the rats excreted an excess of nicotinic acid. This was confirmed in the subsequent experiments with nicotinic acid-free diets, described below.

When the daily food allowance was reduced by one-half for 4 days, the trigonelline fraction was reduced still further to 86 γ. After 5 days of complete fasting the level of both fractions was reduced to uniformly low values. The total of both fractions was then 90 γ per rat per day. While the animals lost on the average 10 gm. in weight during this fasting period, there was apparently some conservation of the nicotinic acid stores, rather than loss, with the attendant tissue breakdown. The levels reached on 5 days of fasting were not as low as those subsequently attained when the rats were placed on a protein-free diet (Table II), but it is clear that in the rat the major end-product of nicotinic acid metabolism and excretion is trigonelline, as it is in man and in the dog (5, 6), but not in the rabbit.[1]

The effects of relatively small single doses of trigonelline, nicotinuric acid, nicotinamide, and of nicotinic acid, ingested with the food, on the urinary excretion are also shown in Table I. Several interesting aspects, apparently peculiar to the rat, are manifested.

After the ingestion of 1.5 mg. of trigonelline there is in the first 2 or 3 days an increase in the trigonelline fraction of the urine, accounting for 25 to 30 per cent of the ingested dose. In the other species studied (man, dog, rabbit) the ingested trigonelline is almost completely excreted in the first 2 days. There is no evidence under these conditions of trigonelline being stored in the tissues and slowly excreted subsequently.

Ingested nicotinuric acid, which is eliminated completely and unchanged by man, dog, and rabbit, is apparently hydrolyzed and

[1] Huff, J. W., and Perlzweig, W. A., unpublished data.

otherwise metabolized by the rat. This conclusion is based on the prompt increase in the urinary trigonelline and in the finding that the very large increase in the acid-hydrolyzable fraction (NA) is due not to unchanged nicotinuric acid but to nicotinic acid. Only negligible amounts of nicotinuric acid and possibly of nicotinamide could be found in the urine in the first 2 days following its ingestion.[2] Only after the repeated administration of larger doses of nicotinuric acid did a portion of this compound appear unchanged in the urine of male rats. Female rats appear to have a greater capacity for splitting it than males.

After a single dose of 1.5 mg. of nicotinamide about 30 per cent of it was excreted as trigonelline and about 5 per cent as the unchanged amide. Very little, if any, nicotinuric acid was formed.

Of a 3 mg. dose of nicotinic acid the rats excreted about 30 per cent as trigonelline and about 7 per cent as the unchanged acid in the urine the first 2 days. Here again, only very small amounts of nicotinuric acid and possibly of the amide were excreted.

In an attempt to facilitate the synthesis of nicotinuric acid glycine was fed together with the nicotinic acid. The effect upon the fractions found in the urine was almost exactly the same as after feeding nicotinic acid alone. No increase in the small nicotinuric acid component could be found.

Synthesis of Nicotinic Acid in Rat and Dietary Factors Affecting It

In order to study the synthesis of nicotinic acid in the rat by means of metabolism experiments it became clear that it was necessary to resort to protein-free diets.

Guggenheim in the second edition of his excellent monograph (7) postulates the following hypothesis concerning the biosynthesis of nicotinic acid.

$$\begin{matrix}\text{Ornithine}\\ \downarrow\uparrow \\ \text{proline}\end{matrix} \underset{+\mathrm{H_2}}{\overset{-\mathrm{NH_2}}{\rightleftharpoons}} \delta\text{-aminovaleric acid} \xrightarrow{-\mathrm{H_2}}$$

$$\delta\text{-aminodehydrovaleric acid} \xrightarrow[-\mathrm{H_2O}]{+\mathrm{CH_2O}} \text{guvacin} \xrightarrow{-\mathrm{H_2}} \text{nicotinic acid}$$

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[2] The method of approximate estimation of the components of the acid-hydrolyzable fraction, by means of comparison of the color values obtained from unhydrolyzed urine with those from urine hydrolyzed with 1 and 6 N HCl, is described in a preceding paper (5).

This hypothesis furnished a general approach to the problem of metabolic experiments in rats.

TABLE II

Effect of Protein-Free Diet and of Addition of Nitrogen Compounds to This Diet on Urinary Excretion of Nicotinic Acid Derivatives

The values are given in micrograms per rat per day.

Day	Group A, 4 females				Group B, 4 males				Group C, 4 males			
	NA*	Trigonelline	Total†	Weight	NA*	Trigonelline	Total†	Weight	NA*	Trigonelline	Total†	Weight
For comparison, urine sample from corn-meal + casein diet (Table I)												
	25	131	143		35	108	132		25	68	86	
Protein-free diet after 3 days fasting												
				gm.				*gm.*				*gm.*
4	23	52	70	301	29	46	70	352	25	114	128	314
5	21	36	53		27	49	71		26	84	102	
6	19	51	65		31	42	69		30	97	117	
7	18	53	66	300	26	39	61	345	24	54	73	309
	25 mg. choline daily, 4 days				100 mg. *dl*-δ-amino-*n*-valeric acid daily, 4 days				1 gm. glycine daily, 4 days			
8	24	92	107		30	97	117		25	117	130	
9	24	112	125		32	112	133		24	134	145	
10	18	131	136		28	152	165		23	140	149	
11	32	141	159		35	205	220		44	143	173	
Protein-free diet												
12	18	77	88		32	123	143		20	91	102	
13	20	103	113		23	77	93		22	77	92	
14	21	123	132	280	24	92	107	331	27	96	114	288

* NA includes all nicotinic acid derivatives hydrolyzable by boiling with 6 N HCl.

† Total represents the sum of NA and trigonelline calculated in terms of nicotinic acid.

The three groups of four rats each used in the above series of experiments were placed on a protein-free and nicotinic acid-free

diet, the basal levels of nicotinic acid excretion established, and the effect of administering orally and parenterally various nitrogenous compounds and protein on the excretion were studied.

The diet consisted of corn-starch 18, cottonseed oil 1, cod liver oil 0.4, $CaCO_3$ 0.2, $CaHPO_4$ 0.2, NaCl 0.2, and water 80 per cent. The corn-starch was mixed to a thick paste with water and poured into an excess of boiling water containing the salts. The starch solution was then autoclaved at 15 pounds for 30 minutes. While the solution was still warm, the oils and the vitamin B supplements were mixed in to produce a homogeneous mass which solidified on cooling to a gel. The vitamin B supplement added was sufficient to provide 15 γ of thiamine, 10 γ of riboflavin, and 15 γ of pyridoxine per rat per day. By analysis, the corn-starch used in this diet contained 0.5 γ of nicotinic acid per gm.

The rats consumed on the average 75 gm. of this diet per day. In the first 21 days of this régime they lost 20 to 30 gm. in weight.

After the first 4 days on the protein-free diet, following 3 days of fasting, the rats reached a fairly constant basal level of excretion of total nicotinic acid derivatives in the urine, the average for the three groups being 67 γ per rat per day (Table II) on an estimated intake of approximately 7 γ of nicotinic acid in the food. Of this amount the trigonelline constituted 60 to 70 per cent, the remainder being the acid-hydrolyzable fraction (NA). This level of urinary excretion was about one-half of that found when these rats were on the casein+corn-meal diet. While the feces were not analyzed in this experiment, the analyses given in Tables IV and V on other rats on the same diet indicate additional excretion in the feces of 40 to 90 γ per rat per day. The total excretion of all determined nicotinic acid derivatives in the urine and feces in the adult rat on a protein-free, nicotinic acid-free diet ranged in our experiments from 70 to 165 γ, expressed as nicotinic acid.

The effect of adding three different sources of nitrogen was then tried: to the food of Group A, 25 mg. of choline (as the hydrochloride), a basic amine which may also serve as a donor of the methyl group; to Group B, 100 mg. of *dl*-δ-amino-*n*-valeric acid, because of the possible rôle assigned to this amino acid in Guggenheim's hypothesis of the synthesis of nicotinic acid referred to above; and to Group C, 1 gm. of glycine as the simplest of the amino acids and as an abundant source of conjugation with nico-

tinic acid to form nicotinuric acid. These additions were made for 4 days, and the urine data are shown in Table II. The results were immediate and striking. On the very 1st day the trigonelline excretion in the rats of all three groups was increased by choline 74, by δ-aminovaleric acid 149, and by glycine 117 per cent, while the acid-hydrolyzable fraction (NA) remained practically unchanged. These increases in the trigonelline fraction in the urine of the three groups progressed for the 4 days as long as the nitrogen compounds were fed, and decreased at once, almost as spectacularly, when the additions were discontinued. It will be observed that the largest effect was produced by feeding 100 mg. of δ-aminovaleric acid, larger than that produced by feeding 1 gm. of glycine; and 25 mg. of choline had almost the same effect as 1 gm. of glycine. The qualitative and quantitative significance of these results requires further extensive investigation. It is clear, however, that the feeding of choline did not result in more extensive methylation of the nicotinic acid, nor did the feeding of glycine cause an increased excretion of nicotinuric acid at the expense of the trigonelline. As was stated above, the power of the rat to form nicotinuric acid appears to be greatly limited, or it may be split to nicotinic acid as it is formed.

The addition to the food of 1 gm. of casein per rat per day for 7 days resulted in an increased trigonelline excretion in the two groups of male rats, Groups B and C, but not in the female rats of Group A (Table III). Here also, as in respect to the splitting of nicotinuric acid, the anomalous behavior of the females cannot be accounted for and will be investigated further. The removal of the casein from the diet brought an immediate and significant reduction of the trigonelline in the urine of the male rats.

Although the prompt response, within 24 hours, in the excretion of trigonelline following the addition to and the removal from the food of choline, δ-aminovaleric acid, glycine, and casein, more or less precluded the possibility of the synthesis of the extra nicotinic acid being due to the bacterial flora of the gut, at least as the chief factor, it was believed desirable to verify this point further by means of parenteral administration of the nitrogenous compounds and by including the analyses of the feces.

Table IV shows the results of an experiment with the intraperitoneal administration of glycine in which a group of three adult

male rats was used. A preliminary period of 4 days of fasting followed by 3 days of the protein-free diet brought the urinary excretion on the 3rd day of the diet to a low level of 21 γ of acid-hydrolyzable nicotinic acid and 13 γ of trigonelline per rat. The

TABLE III

Effect of Adding Casein to Protein-Free Diet

Same animals as in Table II. The values are given in micrograms per rat per day.

Day	Group A, 4 females			Group B, 4 males			Group C, 4 males		
	NA*	Trigonelline	Total†	NA*	Trigonelline	Total†	NA*	Trigonelline	Total†
Basal level, from Table II, Day 7									
	18	53	66	26	39	61	24	54	73
Protein-free diet + 1 gm. casein per rat per day									
1	18	63	74	21	52	68	16	46	57
2	18	51	64	30	180	191	24	108	121
3	23	77	93	29	227	233	33	165	180
4	27	71	91	33	131	150	39	210	228
6	28	67	88	36	147	168	47	265	283
7	19	61	74	34	183	198	39	245	259
Protein-free diet only									
8	30	77	99	35	150	170	34	172	188
9	29	76	97	32	96	118	31	72	96
10	25	38	59						
11				26	35	57	21	30	48

* NA includes all nicotinic acid derivatives hydrolyzable by boiling with 6 N HCl.

† Total represents the sum of NA and trigonelline calculated in terms of nicotinic acid.

fecal excretion was somewhat higher, amounting to 56 γ of NA and 13 γ of trigonelline.

The daily intraperitoneal injection of 0.6 gm. of glycine for 3 days resulted within 24 hours in a 4-fold increase in the urinary trigonelline, but not in the NA fraction in the urine. On the next day of glycine treatment the urinary trigonelline rose still higher. Neither the NA nor the trigonelline fractions in the feces were ap-

preciably affected. When the glycine was discontinued, the urinary trigonelline dropped promptly to the initial low level.

It is thus manifested that the intestinal flora plays a slight, if any, rôle in the synthesis of nicotinic acid in the rat organism. It is to be noted in Tables IV to VI that in the feces the NA fraction is always considerably higher than the trigonelline fraction, the latter occasionally being vanishingly small. Nor are the fluctua-

TABLE IV

Effect of Intraperitoneal Glycine Injections on Group H (Three Male Rats)

The values are given in micrograms per rat per day.

Day	Urine			Feces		
	NA*	Trigonelline	Total†	NA*	Trigonelline	Total†
Protein-free diet after 4 days fasting						
1				33	11	43
2	17	12	28	45	16	59
3	21	13	33	56	13	68
Same diet + 0.6 gm. glycine intraperitoneally daily						
4	22	59	75	47	13	59
5	23	101	114	62	11	72
6	25	68	86	60	19	77
Discontinued glycine						
7	16	14	29	65	9	73
8	17	18	34	44	14	57

* NA includes all nicotinic acid derivatives hydrolyzable by boiling with 6 N HCl.

† Total represents the sum of NA and trigonelline calculated in terms of nicotinic acid.

tions in the total nicotinic acid excretion in the feces from day to day as great as in the urine. The different pattern of the fractions and the relative constancy of the total amount excreted indicate that the intestinal bacteria synthesize nicotinic acid and metabolize it in a different way from the body tissues.

Among other nitrogenous compounds, ammonium lactate was selected as the simplest possible source of nitrogen. Accordingly, 1.67 gm. of this compound, prepared by the neutralization of lactic

acid with concentrated ammonia, were added daily to the protein-free diet of each of three male rats. As will be seen in Table V, 3 days of feeding ammonium lactate failed to influence appreciably the nicotinic acid excretion in the urine and in the feces.

TABLE V

Effect of Ammonium Lactate, Administered Orally and Intraperitoneally, on Group I (Three Male Rats)

The values are given in micrograms per rat per day.

Day	Urine			Feces		
	NA*	Trigonelline	Total†	NA*	Trigonelline	Total†
Protein-free diet after 4 days fasting						
2	14	12	25			
3	17	14	30	75	14	88
Same diet + 1.67 gm. ammonium lactate orally, daily						
5	24	28	49			
6	20	16	34	88	3	91
Discontinued ammonium lactate, same diet						
8	20	30	47			
10	20	42	58	70	8	77
12	24	75	91	68	8	75
Same diet + 2.10 gm. ammonium lactate intraperitoneally, daily						
13	21	165	169	29	2	31
14	32	164	180			
16	22	219	219	35	5	40
Discontinued ammonium lactate, same diet						
17	17	29	43	40	4	44
19	16	32	45	54	6	59

* NA includes all nicotinic acid derivatives hydrolyzable by boiling with 6 N HCl.

† Total represents the sum of NA and trigonelline calculated in terms of nicotinic acid.

After a rest period of 4 days on the protein-free diet, 2.1 gm. of ammonium lactate, divided into three doses of 0.7 gm. each dissolved in 2 cc. of water, were injected intraperitoneally into each rat daily for 4 days. The increase in the trigonelline excretion in the urine was immediate and much greater than that obtained with

the injection of glycine in the preceding experiment. When the administration of the lactate was discontinued, the urinary trigonelline dropped promptly to the basal level. The fecal excretion of both nicotinic acid and of trigonelline was oddly enough somewhat decreased after the injection of the lactate, and rose again after it was discontinued.

The difference in the response of the rats after oral and parenteral administration of ammonium lactate suggests the importance of the channels, and perhaps of the rate, of supply of the components involved in the synthesis of nicotinic acid.

Oral administration of relatively large amounts of urea did not produce an increase of nicotinic acid excretion in the rats. Experiments with the parenteral administration of urea and of other nitrogenous compounds are being conducted and will be reported in a later communication.

An attempt to reduce the bacterial synthesis of nicotinic acid in rats by feeding of sulfaguanidine was not unequivocally successful, because of the toxicity of the large amounts of the drug required to sterilize the gut.

The above data suggest strongly that the tissues of the rat are capable of synthesizing nicotinic acid from the simplest of ammonium salts, amines, and amino acids, and that any contribution of the intestinal bacteria to this synthesis is of a small order of magnitude.

Attempt to Saturate Rat with Nicotinic Acid

In the experiments described above not more than 50 per cent of ingested nicotinic acid could be recovered from the urine and feces of rats when the amount of nicotinic acid or of the amide given was at the level of 10 mg. per kilo of body weight. In one experiment this amount of amide was administered to a group of rats over a period of 10 days without increasing the rate of excretion above 50 per cent. In order to subject the problems of storage and of the disposal of nicotinic acid in the rat to a more rigorous test, it was decided to attempt saturation by means of administering it in much higher doses.

Nicotinic acid was mixed with the protein-free diet to six adult male rats, average weight 250 gm., during 7 days at the rate of 1 gm. per kilo of body weight per day. The animals ingested the

food readily and showed no toxic symptoms or loss of appetite throughout this period. The average loss in weight of 16 gm. could be ascribed, from our previous data, to the effect of the protein-free diet alone. The urines and feces were collected and analyzed daily. At the end of the 7 day period the animals were sacrificed, the entire bodies of two rats were repeatedly minced in a fine food chopper, and three weighed 5 gm. aliquots of each were ground with 40 to 50 cc. of water in a mortar and emulsified in a high speed Waring mixer. After the addition of 20 cc. of concentrated HCl the mixtures were heated for 2 hours in a boiling water bath, and on cooling made up to 100 cc. The mixtures were then analyzed for the acid-hydrolyzable NA and trigonelline. The bodies of two control rats kept on the same diet for the same period were similarly analyzed.

Table VI

Distribution of Nicotinic Acid in Excreta and Bodies of Six Rats after 7 Days Ingestion of 1 Gm. per Kilo of Body Weight

The results are the mean values per rat for the entire period.

		NA	Trigonelline	Total
		mg.	*mg.*	*mg.*
Urine	Experimental	725	242	943
	Controls	0.12	0.43	0.5
	Excess in experimental animal			942.5
Feces	Experimental	20.3	0	20.3
	Controls	0.4	0.2	0.6
	Excess in experimental animal			19.7
Bodies	Experimental	24.2	5.5	29.2
	Controls	11.8	1.0	12.7
	Excess in experimental animal			76.5
Total excess in excreta and body				979 (62%)
" nicotinic acid ingested				1570
" unaccounted for				597 (38%)

Table VI presents a summary of the mean values obtained per rat for the period.

It appears from these data that of the 1570 mg. of nicotinic acid ingested about 62 per cent could be accounted for by the excess excretion, almost entirely in the urine, and the small amount stored

in the body. The excess excreted in the urine consisted of largely unchanged nicotinic acid; the glycine conjugate and the amide were present, if at all, in very small amounts, as indicated by the method of fractional hydrolysis. Under excessive loading with nicotinic acid, 225 mg. per rat, the limit of methylation appeared to be reached with the maximum value observed of 56 mg. of trigonelline. A relatively small amount, a little over 1 per cent, was lost in the feces, entirely as unchanged nicotinic acid, indicating that most of the ingested nicotinic acid was absorbed if allowance is made for a possibility of some destruction by the intestinal bacteria.

About 38 per cent of the nicotinic acid remains unaccounted for. No evidence could be found from the total nitrogen and urea analyses of the urines that any significant amounts of the nicotinic acid were transformed into urea or other nitrogenous compounds which are detected by the Kjeldahl process. Nor could any pyridine bases be detected by extraction and distillation procedures. It seems probable, therefore, that some of the nicotinic acid was transformed into pyridine derivatives which do not react with the CNBr-amine reagents employed in these studies. This provocative problem, also encountered in man, dog, and rabbit, is being investigated further.

SUMMARY

The chief end-product of nicotinic acid metabolism in the rat is trigonelline, as it is in man and in the dog.

On a diet providing about 5 gm. of protein (from casein and corn-meal) an adult rat of 250 to 350 gm. excretes on the average 120 γ of nicotinic acid and its derivatives (including 100 γ as trigonelline) in the urine, which corresponds closely to the intake of nicotinic acid in the food. After 5 days of fasting the total daily nicotinic acid excretion in the urine dropped to 90 γ per animal.

Trigonelline when administered orally in 1.5 mg. doses is excreted unchanged in the urine to the extent of 20 to 40 per cent, which is much less than the proportion excreted by man, the dog, or the rabbit. The fate of the rest of the trigonelline remains undetermined.

Nicotinuric acid when administered orally in doses equivalent to 1.5 to 3.0 mg. of nicotinic acid is excreted partly unchanged, but a

substantial part appears as free nicotinic acid and as trigonelline. The rat, unlike the other species studied, possesses a mechanism for splitting nicotinuric acid.

Nicotinamide given orally in 1.5 mg. doses is excreted in the urine to the extent of 30 per cent in 24 hours, largely as trigonelline; a small amount appears unchanged as the amide.

Nicotinic acid fed in 3 mg. doses is excreted in the 24 hour urine also to the extent of about 30 per cent of the ingested dose, of which 70 to 80 per cent is trigonelline, the remainder consisting of unchanged nicotinic acid and small amounts of nicotinuric acid and amide. The simultaneous administration of large amounts of glycine with the nicotinic acid does not lead to increased nicotinuric acid excretion.

On a protein-free diet containing 7 γ of nicotinic acid adult rats excrete daily a total of 25 to 75 γ of nicotinic acid derivatives in the urine, and 40 to 90 γ in the feces.

On the addition of casein, amino acids (glycine and *dl*-δ-amino-*n*-valeric acid), or choline to the protein-free diet the urinary, but not the fecal, excretion of nicotinic acid derivatives is promptly and significantly increased, and decreases to original levels on the withdrawal of the nitrogenous compounds.

These results are obtained after the parenteral as well as after the oral administration of the nitrogen compounds. Ammonium lactate given intraperitoneally also increases the excretion of nicotinic acid.

These observations may be interpreted as evidence for the synthesis of nicotinic acid in the tissues of the rat, apart from any bacterial synthesis which occurs in the gut.

After massive doses of nicotinic acid, 1 gm. per kilo, are fed for 7 days to six adult rats, two-thirds of the total amount administered was recovered from the urines, while insignificant amounts were found in the feces and stored in the bodies. The one-third of the nicotinic acid thus unaccounted for could not be found in the other nitrogenous components of the urine.

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THE QUANTITATIVE DETERMINATION OF CYTOCHROME C*

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In the course of a quantitative study of the distribution of cytochrome *c* in cancer tissue a consideration of the available methods of analysis led to the development of an improved method which has been applied to this particular study.[1] The method has been used under such a variety of conditions that it is believed that it will give satisfactory results if applied to other problems involving the concentration of cytochrome *c* in animal tissues.

Both manometric and spectrophotometric techniques have been applied to the determination of cytochrome *c* by previous investigators. Von Euler and Hellström (2) reported the cytochrome *c* content of Jensen rat sarcoma on the basis of a qualitative spectrophotometric method. Junowicz-Kocholaty and Hogness (3) have presented a method for cytochrome *c* determination which is essentially Keilin and Hartree's method for the preparation of pure cytochrome *c* (4) placed on a quantitative basis. The method requires the use of 100 gm. of tissue, a quantity too large for experiments in which rats and mice are used as experimental animals. Fujita, Hata, and Numata (5) presented a spectrophotometric method for the determination of cytochrome *c* in which an attempt was made to isolate the cytochrome in pure form. Attempts to recover added cytochrome *c* by this method gave unsatisfactory results. It is believed that an incomplete precipitation of cytochrome *c* occurred during the acetone treatment.

By far the most satisfactory method in the literature thus far is

* This work was aided by the Jonathan Bowman Fund for Cancer Research.

[1] A preliminary report of the results of this study has appeared (1).

that of Stotz (6) whose method is based on the catalytic function of cytochrome. The determination is made by comparing the rate of oxygen uptake obtained with an unknown solution and that obtained with a standard cytochrome *c* solution. However, since extraneous factors present a constant danger in the case of analyses based on rate measurements, we attempted to devise an absolute measurement of the cytochrome *c* concentration. This can be accomplished spectrophotometrically by employing the absorption coefficients for reduced and oxidized cytochrome *c*. Potter (7) recently suggested that it should be possible to determine cytochrome *c* spectrophotometrically by means of the enzymatic oxidation and subsequent reduction of the compound, since he obtained 100 per cent recovery of added cytochrome *c* when the reduction was carried out in the presence of 0.001 M cyanide added after succinate. It seemed that a specific enzymatic spectrophotometric measurement such as this would eliminate difficulties encountered in rate measurement and would also eliminate the interference of hemoglobin (3), thus eliminating the necessity for a hemoglobin-free extract.

The method which we are presenting for the determination of cytochrome *c* in animal tissues is based on the following three facts: (*a*) The absorption of reduced cytochrome *c* is much greater than the absorption of oxidized cytochrome *c* at a wave-length of 550 mμ. (*b*) The total absorption of a solution containing several solutes is equal to the sum of their individual absorptions in the absence of interaction. (*c*) Cytochrome *c* can be specifically oxidized and reduced by enzymatic means without altering the absorption of other components of the test solution.

Method

Extraction and Concentration—Weighed tissues are homogenized in 5 to 20 volumes of distilled water according to the Potter and Elvehjem technique (8) with a stainless steel pestle. The homogenate is quantitatively transferred to a beaker and the pH is adjusted to 3.5 by addition of 3 per cent trichloroacetic acid with a glass electrode to measure the acidity. The mixture is allowed to stand for 1½ hours to extract the cytochrome *c* (3, 4, 6). The mixture is then transferred to graduated conical tubes and centrifuged for 10 minutes at 3000 R.P.M. Since the cytochrome is dis-

tributed between the residue and the supernatant, the volumes of both are recorded for subsequent calculations. The supernatant fluid is brought to pH 7.0 with 1 per cent sodium hydroxide, and after standing 5 to 10 minutes the mixture is again centrifuged at 3000 R.P.M. for 10 minutes. The precipitate should be relatively small at this point, and is discarded. The supernatant fluid is poured into graduated centrifuge tubes and a quantity of 100 per cent trichloroacetic acid solution equal to 8 per cent of the volume of supernatant fluid is added. The mixture is allowed to stand for 20 minutes and is then centrifuged for 10 minutes at 3000 R.P.M. The supernatant liquid is carefully removed and discarded. The precipitate containing the cytochrome *c*, which usually occupies about 0.20 ml. when packed, is taken up in a quantity of water less than 2 ml. and dissolved in a drop of 2 N sodium hydroxide, plus further additions of 0.1 N alkali as needed. Any excess sodium hydroxide is neutralized by addition of 0.1 N hydrochloric acid. Here an external indicator, phenol red, is used to determine when the solution is neutral. The unknown solution is finally diluted to a volume of 2.5 ml. with distilled water.

Spectrophotometric Measurement—The estimation of the cytochrome *c* content of the unknown solution is carried out with the use of a photoelectric spectrophotometer (7, 9). To each sample 0.3 ml. of 0.25 M phosphate buffer, pH 7.4, and 0.2 ml. of a kidney enzyme preparation containing both succinic dehydrogenase and cytochrome oxidase are added, giving a total volume of 3.0 ml. The unknown solution is next placed in a 1 cm. spectrophotometric cell and the absorption is measured at 550 mμ with a 2.5 mμ exit slit. The action of cytochrome oxidase in the absence of substrates which reduce cytochrome *c* converts the latter to the oxidized form. The extinction observed is due to oxidized cytochrome plus other colored substances present, chiefly flavin and hemin derivatives. Next 0.01 ml. of 0.5 M succinate is added to the cell. After mixing, 0.03 ml. of 0.1 M neutralized cyanide is added. This concentration of cyanide stops the action of the oxidase but does not combine with the cytochrome *c* under these conditions (7). After mixing, the cytochrome *c* is converted to the reduced form within 30 seconds by the action of succinic dehydrogenase, and a second spectrophotometric reading is taken. From the two extinction values obtained the concentration of cytochrome *c* may be

calculated. If C_t equals the total cytochrome *c* in moles per ml., E_r the observed extinction of reduced cytochrome *c*, E_o the observed extinction of oxidized cytochrome *c*, and α for $E_r - E_o$ is 1.91×10^7 (see (7)),[2] then,

$$C_t = \frac{E_r - E_o}{1.91} \times 10^{-7}$$

Since the final solution has a volume of 3 ml., it is necessary to multiply the result by 3 and, in addition, to correct for the volume of the residue after extraction in order to obtain the amount of cytochrome in the sample.

Comments on Method

Extraction—Since the extraction of the cytochrome *c* from the tissue is of primary importance, careful consideration was given to the factors involved in this procedure. In studying the optimum pH for extraction, rat liver and kidney tissues were extracted at acidities varying from pH 3.0 to 4.5. In agreement with the finding of Stotz (6) we found that maximum extraction was obtained at pH 3.5, with an optimum range of about pH 3.2 to 3.8.

Since the effectiveness of extraction cannot be tested by recovery experiments, other lines of evidence must be advanced to test the procedure. That homogenization *per se* disrupts the cells and disperses the cytochrome into the medium is clearly demonstrated by the fact that a dilution effect which can be overcome by the addition of pure cytochrome *c* can be demonstrated in the case of the succinoxidase system in liver (10). This result is obtained in other tissues as well. Further evidence that extraction is complete was shown by reextracting the first residue obtained in the preparation of pure cytochrome *c* from beef heart muscle. By again homogenizing the acid-extracted residue, adjusting to pH 3.5 with trichloroacetic acid, and extracting for $1\frac{1}{2}$ hours it was found that the first extraction was complete; *i.e.*, it was found that as much cytochrome *c* remained in the precipitate as was present in an equal volume of supernatant fluid. A third test was made by increasing the volume of the extraction mixture and holding the amount of tissue constant. It was found that a given volume

[2] The value of the constant should be determined for the particular spectrophotometric conditions used.

of precipitate contained as much cytochrome *c* as an equal volume of supernatant liquid. It is apparent, therefore, that the extraction procedure is effective but that the precipitate volume must always be recorded and considered in calculating the quantity of cytochrome *c* present in any sample.

Neutralization of Acid Extract—In a study of the neutralization of the acid extract it was concluded that this is an essential step in the procedure but evidence was obtained that adsorption of cytochrome *c* on the precipitate took place if the mixture was allowed to stand for longer than the recommended time before centrifugation.

In the special case of rat skeletal muscle poor recovery of added cytochrome was found. However, by adjusting the pH to 5.0 instead of 7.0, 93 per cent recovery of added cytochrome was obtained. The volume of precipitate obtained at this point was decreased by the modification. This suggested that adsorption of cytochrome *c* on the unusually voluminous precipitate obtained in the case of skeletal muscle was the cause of the loss of cytochrome *c*. Homogenizing tissues so thoroughly that the particles are too finely dispersed to be precipitated during the first centrifuging process is to be avoided, since in such a case the precipitate volume after neutralization to pH 7.0 is greater and adsorption of cytochrome *c* may occur. If a tissue is encountered which gives a large precipitate at this step, it is very important that recovery experiments be carried out to test this particular step.

In the analysis of livers containing tumors induced by dimethylaminoazobenzene a difficulty was encountered in that the presence of a fatty constituent rendered the final solution too turbid. It appeared that extraction of the fat-like substance would be necessary before the analysis was made, but by carefully removing the supernatant liquid from the precipitate after the first centrifuging, the fatty substance adhered to the sides of the centrifuge tube and in that manner could be separated from the supernatant liquid.

Quantitative Precipitation of Cytochrome c—Any method for the analysis of cytochrome *c* must involve a step in which the cytochrome in a relatively large volume of extract is concentrated to a small volume. Stotz (6) precipitated the cytochrome with phosphotungstic acid, and then removed the latter with barium in the presence of phosphate to avoid an excess of barium. The final

solution obviously contains a saturated solution of barium phosphate and when this is concentrated *in vacuo* a precipitate of barium phosphate is likely to result. Since cytochrome *c* is adsorbed on barium phosphate, there is considerable danger of losing cytochrome at this step, and we have been unable to recover small amounts of cytochrome. This procedure is likely to give too low results on samples which are originally low in cytochrome.

Since our proposed method would give valid results even in the case of partial inhibitions of rate, we attempted to concentrate the cytochrome by precipitation with phosphotungstic acid and to make the measurement in the presence of the phosphotungstate. This was not feasible, since the latter completely inhibited the enzyme preparation. However, experiments showed that sodium trichloroacetate did not interfere with the enzymatic oxidation and reduction, and trichloroacetic acid was therefore tested as a cytochrome precipitant, since it is known to be very effective as a protein precipitant. Pure samples of cytochrome were used and the analysis was effected spectrophotometrically by chemical reduction with sodium hydrosulfite. It was found that in all samples in which the final concentration of trichloroacetic acid was greater than 5 per cent the cytochrome was completely precipitated. It was further found that a given amount of cytochrome *c* could be quantitatively precipitated from a volume of extract several times larger than is ever encountered in practice. It is thus possible to effect the concentration of cytochrome *c* with trichloroacetic acid and to make the quantitative measurement without removing the precipitant.

Quantity of Tissue Needed—In order to insure a sufficient difference in extinction between the oxidized and reduced cytochrome *c* when determined spectrophotometrically it was necessary to determine the quantity of tissue necessary for the determination. 1 to 2 gm. of normal rat tissues is sufficient, while 5 to 10 gm. of cancer tissue are required. In studying the accuracy of the method various tissues such as rat liver, spleen, and cancer tissue were used in recovery experiments. In all cases we have recovered greater than 93 per cent of added cytochrome *c*.

Although the cyanide concentration has been adjusted to a very low concentration, experiments were designed to make certain that cyanide did not combine with part of the cytochrome *c*, as has been noted (7) in the case of higher concentrations of cyanide.

Small quantities of cytochrome *c* ranging from 0.025 to 0.075 × 10^{-7} mole of cytochrome *c* were added to 1 gm. samples of Flexner-Jobling rat carcinoma,[3] so that the final concentration of cytochrome *c* in each sample was lower than that present in any actual tissue analysis. Recovery between 96 and 100 per cent was obtained in all samples, thus eliminating the possibility of cyanide combining with cytochrome *c* in this analysis, as well as insuring the over-all accuracy of the method.

Method of Preparation of Enzyme—A rat kidney is homogenized in 9 volumes of cold M/30 phosphate buffer (pH 7.4), centrifuged for 4 minutes at 1000 R.P.M., and then filtered through coarse filter paper. Although the cytochrome *c* has not been removed from this preparation, the quantity present in 0.2 ml. cannot be detected spectrophotometrically and thus does not interfere in the determination. The strength of the enzyme must be proved with a sample of pure cytochrome. Reduction should occur within 30 seconds.

Since the determination of cytochrome *c* by this method depends upon measuring the difference in extinction between oxidized and reduced cytochrome *c*, it is essential that the cytochrome *c* in the unknown solution be completely oxidized before the initial reading is taken. There are three steps in this method which insure complete oxidation of cytochrome *c*, (*a*) the precipitation of cytochrome *c* with trichloroacetic acid (3), (*b*) dissolving the trichloroacetic acid precipitate in sodium hydroxide (3), (*c*) the action of the cytochrome oxidase in the final solution. In order to prove that the quantity of cytochrome oxidase used was sufficient, even if all of the cytochrome present were in the reduced form, an experiment was carried out in which 11 γ of ascorbic acid were added to 2 × 10^{-8} mole of cytochrome *c*. This resulted in reduction of about 80 per cent of the cytochrome *c*. Varying quantities of the enzyme preparation were used and as little as 0.01 ml. of the enzyme preparation was found to give complete oxidation in 2 minutes. Since 0.2 ml. of the enzyme preparation is used in actual analyses, assurance of complete oxidation was obtained. The turbidity of the enzyme does not interfere with the method, largely because it is automatically corrected for.

Removal of Hemoglobin—As mentioned previously, the presence

[3] A homogenate of pooled samples was used and the concentration of cytochrome in the tumor was determined on an aliquot equivalent to 5 gm.

of hemoglobin does not ordinarily interfere with the determination. However, in the case of certain tissues such as spleen it is desirable to remove part of the hemoglobin to decrease the total absorption in the spectrophotometric measurement. This can be accomplished by acidifying the final unknown solution to pH 4 to 6 with dilute HCl, which precipitates the hemoglobin. The mixture is centrifuged and the precipitate is washed with 0.1 N HCl. The combined supernatant liquids are again neutralized to pH 7.0.

TABLE I

Cytochrome c Content of Normal Rat Tissues

The results are expressed as micrograms of cytochrome c per gm. of fresh tissue.

Rat No.	Heart	Kidney	Skeletal muscle	Brain	Liver	Spleen	Lung
1	393	266	97	76	85	63	18
2	432	300	94	41	89	45	16
3	379	249	97	59	93	34	30
4	346	264	87	54	90	40	15
5	363	232	91	43	94	30	20
6	412	254	108	41	96	40	23
7	353	235	83	45	87	32	18
8	374	208	97	41	91	32	24
9	316	218	105	58	87	61	28
10	345	245	110	45	92	51	19
Average.........	371	247	97	50	90	43	21
" from Stotz (6).......	530	330	160	75	68	48	29

Recovery of over 90 per cent of the cytochrome c from spleen was obtained.

Application of Method

Distribution of Cytochrome c in Normal Rat Tissues—This method of analysis has been applied to several tissues from normal adult rats. A wide variation in cytochrome c content of different organs was noted, in substantial agreement with the results of Stotz (6). Table I gives the cytochrome c content of the various tissues analyzed. Duplicate samples check within about 5 per cent of the average value but it should be noted that there is

rather wide variation between animals as was also noted by Stotz. The results in Table I were calculated with 16,500 as the molecular weight of cytochrome *c*. The average values obtained by Stotz with the same molecular weight are included for comparison.

It is of interest to note that the levels obtained by Stotz are about 50 per cent higher than ours in the case of tissues high in cytochrome *c*, while they check very closely in the case of lung and spleen, which are low in cytochrome. It is not possible to say whether the differences are in the material or the methods. Since the molecular weight may actually be as low as 11,700 (11) it might be preferable to state the results in moles as given in the method of calculation above.

TABLE II

Cytochrome c Content of Chick Embryo

The results are expressed as micrograms of cytochrome *c* per gm. of fresh tissue.

Age of embryo	No. of samples	Maximum	Minimum	Average
days				
6	4	1	0	0
10	4	4	2	3
12	5	8	5	6
16	9	7	5	6
17	7	8	4	6

Stotz reported that rat embryos were low in cytochrome *c*. We have analyzed a number of chick embryos which are also low in cytochrome *c*, and they show an increase in cytochrome concentration with age, as is shown in Table II. This material is so low in cytochrome *c* as to make the analysis extremely difficult, as well as of questionable accuracy as far as comparative studies on embryos of a given age are concerned. It would be of considerable interest to determine whether the cytochrome *c* concentration in very young rats is inversely related to their recently demonstrated ability (12) to withstand short periods of anaerobiosis.

SUMMARY

1. A new method for the determination of cytochrome *c* in small quantities of tissue from experimental animals has been developed.

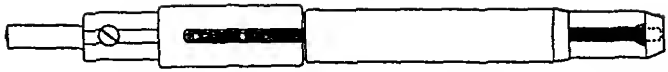

FIG. 1. Instrument for quick removal of tissue. *A* is a rod which fits a hand drill-chuck. It is fastened with a screw into a steel pipe (*B*) having a cutting, sharp opening at the other end and a milled-out groove for a flexible steel spring knife (black) with sharp side and end edges. The latter can be pushed across the pipe by the movable sleeve (*C*) and is covered by the fixed sleeve (*D*). Before the piece is cut out, the steel spring knife is pulled back. The pipe is drilled into the tissue. The spring knife is pushed across the opening and the pipe rotated and pulled out. The sleeve (*C*) is pulled back and the tissue cylinder taken out with forceps.

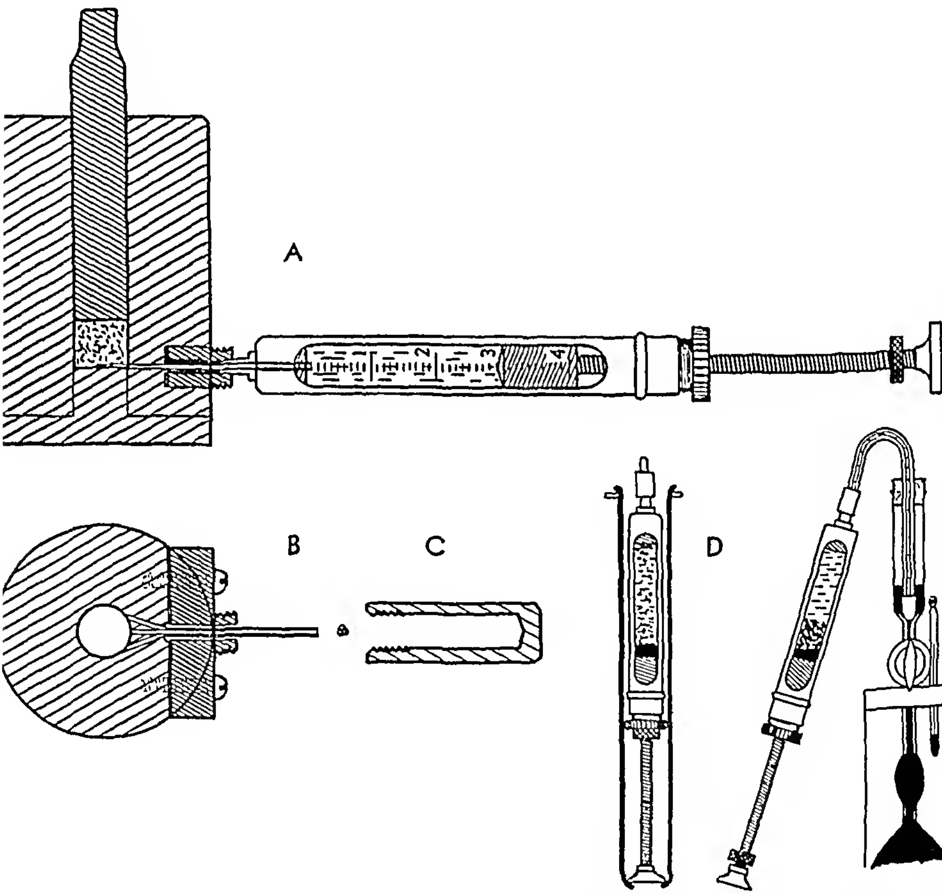

FIG. 2. Device for analyzing the gas content of tissue. *A* represents the syringe with extracted ferricyanide solution attached to the nozzle of the press, the steel cannulas protruding freely into the solution; *B*, cross-section of the press showing the arrangement of the three steel cannulas and the nozzle block fitting into a milled-out slot of the press; *C*, cap to be screwed on the nozzle for the protection of the cannulas; *D*, syringe placed in the centrifuge tube with the piston arrested by a nut; *E*, anaerobic transfer of solution from the syringe to the Van Slyke apparatus or *vice versa*. The press is reproduced one-half the actual size.

the syringe is closed with a rubber cap. After the tissue threads are thoroughly mixed and broken up, the syringe is placed in a stand with its tip upwards and with the piston stem nut tightened as in Fig. 2, *D*. After 10 to 20 minutes equilibration the syringe is put into a special centrifuge tube holder (Fig. 2, *D*) which fits on an ordinary small hand centrifuge. The syringe is centrifuged at a moderate speed for some minutes and 2 cc. of the clear solution over the tissue débris are anaerobically transferred to the Van Slyke apparatus (Fig. 2, *E*), and are analyzed for carbon dioxide and oxygen (nitrogen) in the usual way, the 0.5 cc. mark on the burette being used for all readings.

TABLE I

Factors for Calculation of Tissue Gases in Volumes Per Cent

0.5 cc. of tissue + 3 cc. of ferricyanide; 2 cc. of solution used in the Van Slyke apparatus; pressure read at the 0.5 cc. mark on the burette.

Temperature	CO_2	O_2 or N_2	Temperature	CO_2	O_2 or N_2
°C.			*°C.*		
15	0.236	0.218	23	0.227	0.212
16	0.235	0.217	24	0.226	0.211
17	0.234	0.216	25	0.225	0.210
18	0.233	0.215	26	0.224	0.210
19	0.231	0.215	27	0.223	0.209
20	0.230	0.214	28	0.222	0.208
21	0.229	0.213	29	0.221	0.207
22	0.228	0.212	30	0.221	0.207

The values were calculated according to Formula 3 of Peters and Van Slyke (2).

In the determination of the oxygen capacity of tissue, *e.g.* muscles, the piece of muscle is pressed out as fine threads directly over a Petri dish. The dish is covered with a wet filter paper on a glass plate. After the muscle hemoglobin is saturated with oxygen, the threads are scraped together into a ball which is placed in the press. 0.5 cc. is pressed out into 3 cc. of extracted neutral ferricyanide solution, any gas bubbles being removed; mercury is run in, the syringe closed, and its contents mixed, equilibrated, centrifuged, and analyzed as described above.

The *C* correction is determined by repeated extraction of 6 cc. of ferricyanide solution, by transferring 3 cc. of it anaerobically

to the syringe (*cf.* Fig. 2, *E*), fitting the syringe to the nozzle, removing it, mixing with mercury, and transferring 2 cc. into the Van Slyke apparatus.

In order to remove the piston from the press, the piston is fastened in the vise and the press pulled by hand. The channels of the press are cleaned by a steel wire after each analysis.

Calculation—It is assumed that the 2 cc. of extract analyzed contain two-thirds of the tissue gases in the 3 cc. of suspension in the syringe. To give the volumes per cent of gas in the tissue the pressure (in mm.) measured with the gas at 0.5 cc. volume, minus the mm. of the *C* correction, is multiplied with the appropriate factor from Table I.

The accuracy of the method is limited, especially by the uneven composition of the tissue. In seal muscle the oxygen content was found to be of the order of 5 volumes per cent with an agreement between duplicate samples of about ±0.5 per cent. The carbon dioxide content was of the order of 30 ± 2 volumes per cent.

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AEROBIC AND ANAEROBIC CHANGES IN SEAL MUSCLES DURING DIVING

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During the course of a dive which may last for 15 or 20 minutes a seal is dependent upon its stores of oxygen and the anaerobic reactions which can proceed in its tissues. The course of utilization of the oxygen store in the lungs and hemoglobin of the blood is regular and uniform throughout the dive (Scholander, 1940; Irving, Scholander, and Grinnell, 1942). In seal muscles the dark red color indicates a further considerable supply of muscle hemoglobin, as has already been observed (Theorell, 1934; Robinson, 1939). The oxygen capacity of the minced seal muscle was found to be from 4 to 6 per cent, which would thus be able to contribute an appreciable reserve for aerobic metabolism during diving, and in the red pectoral muscles of penguins, which contained about 4 volumes per cent of oxygen, the store was consumed during a 5 minute dive (Scholander, 1940). In the resting cat soleus muscle the muscle hemoglobin was reduced within 2 minutes when the artery was clamped (Millikan, 1937, 1939). No determinations have hitherto been made upon the actual rate of reduction of seal muscles during diving.

As to the course of glycolysis during diving, it has been shown that the lactic acid content of rat leg muscles increased greatly during submersion, but not if the animals were narcotized to prevent struggling (Scholander, 1940). But in the blood of seals the concentration of lactic acid did not increase during diving, nor was there any increase in the blood of rats, ducks, and penguins (Scholander, 1940). Likewise in the blood of diving manatees (Scholander and Irving, 1941), in two- and three-toed sloths, armadillos, and iguanas (Irving and Scholander, unpublished ob-

servations), there was not much increase in lactic acid content until after the dive, when suddenly a flood of lactic acid appeared in the blood. It is thus indicated as a general process that lactic acid which is formed during diving does not escape into the blood until recovery commences. This indicated that the muscles are isolated from exchange with the blood during diving and fits in with thermoelectric observations which indicated reduced blood flow through the muscles during arrested breathing (Irving, 1939) or diving (Grinnell, Irving, and Scholander, 1942).

The seal muscles during diving seemed therefore to depend upon their intrinsic reserves. In this situation the utilization of oxygen from the stores of muscle hemoglobin and the progress of glycolysis would proceed without external interference. The progress of the aerobic and glycolytic reactions can be followed during the prolonged experimental dives which the seal will endure, and the quantities of oxygen and lactic acid are large enough for accurate determination. The relation of these aerobic and anaerobic reactions in the muscles of diving seals is presented in the following experiments.

Methods

For the present investigation young seals (*Phoca vitulina*), weighing about 20 to 25 kilos, were used. The animals were fastened to a board and placed in a bathtub. Diving was accomplished by lowering the board with the seal under water, leaving only the back of the animal exposed above water. The method used for taking the muscle samples anaerobically and for the analysis of their gas content has been fully described elsewhere (Scholander, 1940, 1942) and only the essential procedure is outlined here. By means of a specially constructed tube a small piece of back muscle is removed through the skin in a few seconds and placed in a micro press which anaerobically spurts a measured part (around 0.5 cc.) of the finely crushed muscle into a syringe filled with extracted ferricyanide solution. After equilibration with the muscle débris the ferricyanide solution is analyzed for gases in the Van Slyke apparatus. Contamination of the muscle piece with blood enters the determination as a source of error. The small amount of blood which may remain in the piece is, however, for the most part squeezed out in the press and dis-

carded before the analysis. Even 5 per cent contamination with blood of 10 volumes per cent oxygen would not affect the determination of the muscle oxygen by more than 0.3 volume per cent in absolute amount, which would be of little significance in the present connection. Part of each muscle sample is ground with sand in trichloroacetic acid, treated with copper lime in the usual way, and analyzed according to Friedemann, Cotonio, and Shaffer (1927) for lactic acid. The muscle samples were always taken from well separated parts of the back musculature of the seal. The results for eleven different diving experiments are given in Figs. 1 to

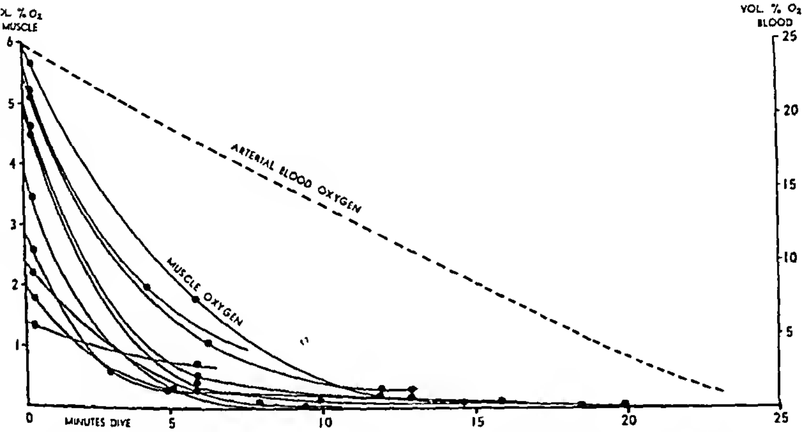

FIG. 1. The oxygen depletion from seal muscles during diving. The average depletion of oxygen from the arterial blood is given for comparison. The dotted circle refers to an inconsistent determination in the series of the upper curve.

3, where the oxygen content, the carbon dioxide content, and the lactic acid content are plotted separately against time.

Oxygen Content—The result of the oxygen determination of the seal muscles during diving is seen in Fig. 1, representing ten curves from ten different seals. The average course of the oxygen depletion from the arterial blood during diving is plotted for comparison. The variation of the oxygen content of the muscles in the first samples at the beginning of the dive, from 1.5 to 6 volumes per cent, suggests that at the time of sampling not all of the muscles were thoroughly circulated with blood and saturated with oxygen. This was also clearly visible from the color of the pieces, which varied from

dark to a bright red. During the first 5 to 10 minutes of the dive practically all of the oxygen of the muscles was used up along a course represented by an exponential curve. The muscle hemoglobin was reduced at a time when the pressure of oxygen in the blood was still high. Theorell (1934) found that a solution of horse muscle hemoglobin became half saturated with oxygen at 3.26 mm., whereas the arterial blood in seals is half saturated at 30 to 35 mm. (*in vitro* (Irving, Solandt, Solandt, and Fisher, 1935), *in vivo* (Scholander, 1940)). Since the muscle hemoglobin is practically reduced at a time when the arterial blood is only half reduced, there seems to be a considerable difference in oxygen tension between the muscles and the blood. This situation develops before any appreciable accumulation of lactic acid or carbon dioxide could influence the dissociation of the muscle hemoglobin. The difference in tension appears to be so great as to suggest that there is no circulation in the muscles during the dive.

During recovery the oxygen content of the muscles was usually restored within a few minutes, but sometimes only after some delay. In samples from both the pre-dive period and from the recovery it was visible that the muscles were occasionally oxygenated only in patches, so that only part of the piece might be bright red, which points to an uneven distribution of the circulation.

Carbon Dioxide Content—The carbon dioxide content of the same muscle pieces as were used for the oxygen determination is shown in Fig. 2. The average content of carbon dioxide in the muscles before diving was about 30 to 35 volumes per cent. During diving the carbon dioxide content tended to rise a little and dropped off a few volumes per cent below the starting value in recovery. The rise during the dive was small, amounting to only a few volumes per cent, and agreed with the few volumes per cent of oxygen available for the muscles from the muscle hemoglobin store. A drop of the carbon dioxide content of the muscles in recovery would be expected on account of the 100 to 200 mg. per cent increase of the lactic acid, although the drop would have been bigger if it resulted from a simple displacement of carbon dioxide from bicarbonate.

Lactic Acid Content—The results of the lactic acid determinations on the same samples as were used for the gas determinations

are shown in Fig. 3. At the beginning of the dive the muscles contained less than 30 mg. per cent of lactic acid, except in cases in which the seal had struggled. Not until after around 5 to 10 minutes of diving was there any noticeable increase in the lactic acid, but later there was a smaller or larger rise, apparently de-

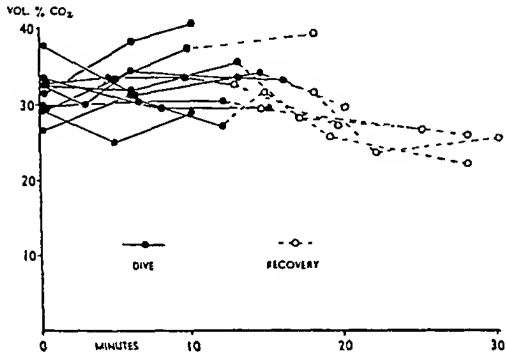

Fig. 2. The carbon dioxide content of seal muscles before, during, and after diving.

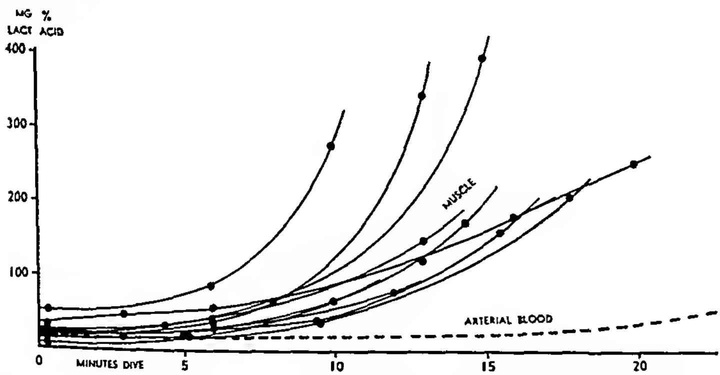

Fig. 3. The lactic acid content in the seal muscles during diving. The average content of lactic acid in the arterial blood is given for comparison.

pending upon how much the seal struggled. The three highest values of Fig. 3 (300 to 400 mg. per cent) occurred after serious struggling. They are of the same order as was previously calculated from the recovery rise of lactic acid in the blood of seals on the assumption of equal distribution between the blood and muscles (Scholander, 1940).

It is of particular interest to note that the time of complete reduction of the muscle hemoglobin coincides with the start of the lactic acid formation in the muscles. The inference is that the presence of the oxygenated muscle hemoglobin prevents the formation of lactic acid. The appearance of lactic acid in the blood first in the recovery after the dive indicates that the muscles are isolated from the circulation during diving.

With the progress of recovery the lactic acid concentration usually decreased, but sometimes rose transiently above the initial recovery value. In all cases in which such a delayed recovery rise was seen in the seal it was, however, combined with a late appearance of oxygenation of the muscles; that is to say, with a prolongation of the anaerobic situation in the muscle. This observation explains satisfactorily the occasional continued rise of lactic acid in recovery of the seal muscle and is the probable explanation for the same phenomenon seen in rats and penguins (Scholander, 1940).

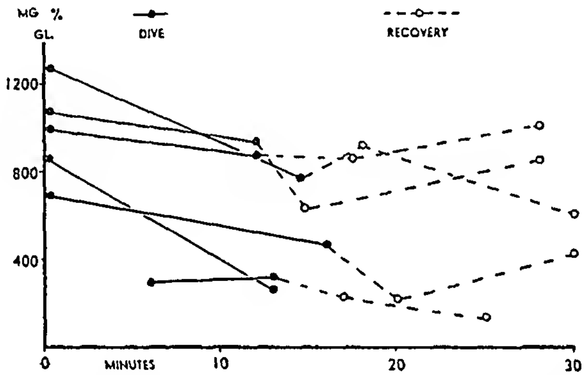

FIG. 4. The glycogen content in seal muscles before, during, and after diving.

Glycogen Content—The result of a few series of glycogen determinations in the muscles before, during, and after the dive is given in Fig. 4. During diving there was found a drop in the glycogen content which was regularly greater than the increase of lactic acid. This was to be expected on account of the aerobic muscle metabolism in the first part of the dive, and may also indicate some accumulation of intermediaries in the course of the breakdown of glycogen to lactic acid. During the time of recovery observed, the glycogen content of the muscles seemed not to be fully restored.

DISCUSSION

If we consider the muscles to be isolated from the circulation during diving for the reasons given above, we can use our figures for the oxygen and lactic acid contents of the muscles to form an estimate of their aerobic and anaerobic metabolism (Table I). The part played by other sources of energy, such as adenylpyrophosphoric acid and creatinephosphoric acid, we do not know. They might raise our figures for anaerobic metabolism by about 30 per cent (*cf.* Lundsgaard (1937)), which in this connection is insignificant. The initial rate of the oxygen depletion of the muscles during diving was about 10 cc. per kilo per minute (Table I, first column), which is probably near the rate of oxygen consumption before the dive. This figure is about 2.5 times the average figure found by Millikan (1937) for the soleus muscle of resting cats, and may in part result from some struggling by the seals when the samples were taken. The estimation suggests that during the first 10 minutes of a dive the total muscular metabolism may drop considerably, but after 10 minutes, as struggling usually increases, the lactic acid concentration usually rises enough so that the total aerobic and anaerobic metabolism for the dive exceeds a non-diving resting metabolism of the same duration. If

TABLE I

Aerobic and Anaerobic Metabolism in Seal Muscle during Diving

Calculated from the average oxygen decrease and lactic acid increase in the muscle during diving, assuming that 5 gm. calories are formed by the consumption of 1 cc. of oxygen and 385 gm. calories from the formation of 1 gm. of lactic acid (*cf.* Evans (1930)).

	Pre-dive	Sections of 20 min. dive			
Time, *min*	5	0–5	5–10	10–15	15–20
Oxygen used per kilo muscle, *cc.*	50	45	3	1	0
Lactic acid formed per kilo muscle, *gm.*	0	0.1	0.35	0 9	1.2
Gm. calories per kilo muscle from oxidation	250	225	15	5	0
Gm. calories per kilo muscle from lactic acid formed	0	39	135	347	462
Gm. calories per kilo muscle from aerobic and anaerobic metabolism	250	264	150	352	462

the muscular activity were entirely cut down or very low, as it is in a dive while the animal is resting or slowly swimming, it is possible that the muscle metabolism would stay lowered for more than 10 minutes. Narcotized rats, unable to struggle, formed scarcely any lactic acid in their muscles when drowned (Scholander, 1940). The general picture is, therefore, as follows: During the first 5 to 10 minutes of the dive the muscle metabolism is aerobic and tends to fall below the pre-dive resting rate. This fall is usually more than compensated for in the latter part of the dive by anaerobic metabolism which is greatly accelerated by any muscular activity; *i.e.*, the total energy expenditure of the muscles is usually greater during diving than before, even in relatively quiet dives.

It seems that the isolation of the muscles from the circulation during diving must limit their capacity for activity because of the small size of the oxygen store and limit for accumulation of lactic acid. It seems doubtful that the lactic acid content could ever surpass 400 mg. per cent. If there were much struggling during the dive, this concentration, which would probably be very painful, would soon be reached and the seal would have to emerge, perhaps even before the oxygen of the blood was very much depleted. At this maximum rate of lactic acid formation in the muscles for a 15 minute dive, their intrinsic metabolism would not provide more than about one-half of the resting metabolism of the whole animal during 15 minutes, even when some allowance for leakage of lactic acid from the muscles is made. What significance the limited muscular metabolism may have for making up the total balance sheet of the diving metabolism will be discussed in another connection (*cf.* Scholander, Irving, and Grinnell (1942)).

It is interesting to note that seals swimming freely or in captivity only rarely make dives of longer duration than 4 to 5 minutes, and thus usually dive under a comfortable aerobic muscular condition without need for acid formation. The anaerobic reserves of the muscles for more prolonged diving must be at least 3 times as great as the aerobic, judged from the maximum diving time and increasing struggles towards the end of a long dive. In the bottlenose whale (*Hyperoodon*) and the sperm-whale (*Physeter*), the oxygen storage capacity of the crushed, nearly black muscles was as large as 6 to 8.5 volumes per cent (Scholander, 1940), or,

in natural conditions, probably closer to 10 volumes per cent, since the oxygen content of seal muscles *in vivo* often was found to be greater than that of the artificially saturated crushed muscles. Inasmuch as the observed diving times in these whales last from $\frac{1}{2}$ to 2 hours, the aerobic resources of the muscles must be supposed to last correspondingly longer than in the seal.

We wish to acknowledge our appreciation of the cooperation of Dr. Paul Galtsoff and the use of the laboratory of the United States Fish and Wildlife Service at Woods Hole. The work was facilitated also by Mr. Thomas H. Dorr of the United States Fish and Wildlife Service at Boothbay Harbor, who supplied the seals. We are indebted to the Rockefeller Foundation for financial support. Miss B. Vennesland kindly made the determination of the glycogen in the muscles.

SUMMARY

By direct gas analyses it was found that the muscle hemoglobin in the diving seal was completely reduced after 5 to 10 minutes diving, after which lactic acid started to form in the muscles. The complete reduction of the muscle hemoglobin at a time when the blood is still half saturated with oxygen indicates isolation of the muscles from the circulation during diving. This is likewise indicated by the fact that the lactic acid which accumulates in the muscles during the dive practically does not enter the blood stream until during the recovery. It is concluded that the muscles are isolated from the circulation during diving and that the muscle hemoglobin acts as a local oxygen store for the muscles, enabling them to carry on aerobically during the first 5 to 10 minutes of a dive without recourse to lactic acid formation until after practically complete reduction of the muscle hemoglobin.

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INFLUENCE OF SOME DIETARY FACTORS ON THE DEVELOPMENT OF RANCIDITY IN THE FAT OF THE WHITE RAT

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(Received for publication, October 23, 1941)

Development of rancidity, particularly of the oxidative type, in fat is one of the important problems in the food industry. Diet influences the type of fatty acids deposited by the animal in its own fat tissue, and this in turn has a marked influence on the speed with which rancidity will develop in the stored fat. There is a possibility that substances which act to prevent oxidation may be present in body fat (5), and that these substances may be carried over from the diet of the animal and be deposited in the fat tissue. Lea (6) says it is "quite reasonable to suppose that nutritional factors may also affect the antioxidant-content of the fatty tissues," and (6) "it might reasonably be expected that changes in fatty-acid composition and possible variations in antioxidant-content might result in some degree of correlation between the susceptibility to oxidation of the fat and the diet of the animal." Lea also observed that under some conditions fat from thin hogs resisted rancidity better than fat from heavier hogs, even though the thin hogs had a softer fat. He suggests that in the heavier hogs fat deposition may have outstripped the accumulation of antioxidants in the tissue (4). In view of these suggestions, it seemed worth while to undertake a study of the influence of feeding antioxidants on the storage life of body fat. Hydroquinone (7, 3) and ascorbic acid (8, 5) have been shown to possess antioxidant activity.

Method

Eighteen rats, 8 weeks old, were divided into three groups. One group received a basal diet only. Each rat in the second group

received the basal diet plus a daily dose of 1 mg. of ascorbic acid, and each rat in the third group received the basal diet plus a daily dose of 1 mg. of hydroquinone. Both the ascorbic acid and the hydroquinone were dissolved in redistilled water, which had been boiled and cooled. The dose was measured accurately in a tuberculin syringe.

The composition of the basal diet was as follows: salt mixture (Osborne and Mendel (9)) 5.00 per cent, casein 14.25, gelatin 0.75, starch 56.00, agar 2.00, yeast concentrate 2.00, cod liver oil 2.00, and olive oil (boiled) 18.00.

The rats were killed after 2 months; the abdominal fat was dissected out and stored in sterile Petri dishes. The fat was stored in the dark, in a cool room. No temperature control was available, but all fats were subject to the same conditions. The fat from two rats from each group was analyzed for rancidity after 4 weeks, and analyses were made on fat from two rats from each series at 2 week intervals thereafter. The Lea peroxide test for rancidity was used (6).

DISCUSSION

Selection of the basic diet was influenced by two considerations. First, it should be as free of antioxidants as possible; second, it should be such that a soft body fat would be deposited, so the course of developing rancidity could be followed in a reasonably short time. The diet reported by Eckstein (2) was used, except that olive oil was substituted for triolein, and cod liver oil was used in place of the non-saponifiable matter from cod liver oil. Eckstein reports that triolein feeding produced a body fat in rats having a higher degree of unsaturation than that obtained with other fats. Olive oil has an oleic acid content close to 85 per cent; so olive oil feeding should result in a soft body fat. Olive oil is also low in antioxidants. In order to reduce the antioxidant content further, the olive oil was boiled in water 1 hour. Banks and Hilditch (1) had observed that olive oil which had been boiled in air-free water in an atmosphere of CO_2 exhibited a much reduced induction period, approximately one-tenth of the original.

Results

The analysis of variance of diet averages alone shows a significant difference in resistance to rancidity due to ascorbic acid

feeding (Table I). F (10)[1] equals 10.53, which approaches the 1 per cent point of 10.56. Since rats do not require ascorbic acid in their food, it may be suggested that the extra feeding of ascorbic acid produced a state more nearly that of tissue saturation, in which sufficient ascorbic acid was retained in the fat tissue to help delay the appearance of rancidity.

The fats showing high rancidity also tended to come from animals showing a high per cent of gain in weight. After correction for the difference due to per cent gain in weight, F equals 4.58 between the ascorbic acid diet and the other diets. This is not quite significant at the 5 per cent point (F equals 5.32 at the 5 per cent point).

TABLE I

Susceptibility to Oxidative Rancidity of Fat from Rats Fed Different Diets

The values are expressed as cc. of 0.002 N sodium thiosulfate per gm. of fat. Each figure represents an average of two samples from each rat.

Diet	Period of storage			Means
	4 wks.	6 wks.	8 wks.	
Basal	10.62	32.14	28.14	21.84
	6.35	46.21	7.58	
" + ascorbic acid	2.23	4.03	6.05	4.71
	1.76	5.19	8.98	
" + hydroquinone	0.44	3.76	66.89	23.92
	4.30	30.71	37.42	

The results indicate that the differences in resistance to rancidity are due partly to thinness or fatness of the animal, and partly to the diet.

Fat from the thinner animals and fat from animals receiving ascorbic acid tended to resist rancidity longer than that from fatter animals, or those receiving the basal diet alone, or basal diet plus hydroquinone.

SUMMARY

Rats were fed a synthetic diet; one group received no supplement, a second group received 1 mg. of ascorbic acid daily, and a third group received 1 mg. of hydroquinone daily.

[1] F, the ratio of two estimates of variance, is tested for significance by comparing the experimental value with tables showing its 5 and 1 per cent points.

The fats from each series were analyzed for rancidity at stated intervals. The results indicate that the differences in resistance to rancidity are due partly to thinness or fatness of the animal, and partly to the diet. Ascorbic acid feeding, together with a low per cent of gain in weight, resulted in a significant increase in resistance of the fat to rancidity.

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THE EFFECT OF GLUCOSE ADMINISTRATION ON THE LEVEL OF BLOOD PANTOTHENIC ACID

Sirs:

Pantothenic acid, first recognized as an essential nutrilite for certain strains of yeast, appears now to function in some fundamental manner in the metabolism of all living matter.

Suggestive data have been obtained that pantothenic acid plays a rôle in carbohydrate metabolism. This work has been reviewed by Williams.[1] More recently Russell and Nasset,[2] using dogs, found that supplementing an adequate stock ration with pantothenic acid resulted in a more rapid disappearance of the carbohydrate component from the gastrointestinal tract. The present report concerns the effect of glucose administration on the level of blood pantothenic acid with a view to determining more specifically the function of pantothenic acid in metabolism.

Adult rabbits previously maintained on a diet of Purina rabbit chow were fasted for 12 hours. They were then fed varying amounts of a 50 per cent aqueous solution of pure glucose by stomach tube. Rabbits receiving corresponding amounts of water by stomach tube as well as untreated rabbits served as controls. At 30 minute intervals after treatment blood samples were drawn from the marginal vein of the ear and the content of pantothenic acid and glucose quantitatively determined. Pantothenic acid was determined by the method of Pennington, Snell, and Williams[3] as modified by Stanbery, Snell, and Spies[4] for application to the determination of pantothenic acid in blood. Blood sugar was determined by the method of Folin and Wu.[5]

[1] Williams, R. J., *Enzymologia*, 9, 387 (1941).

[2] Russell, R. A., and Nasset, E. S., *J. Nutrition*, 22, 287 (1941).

[3] Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 135, 213 (1940).

[4] Stanbery, S. R., Snell, E. E., and Spies, T. D., *J. Biol. Chem.*, 135, 353 (1940).

[5] Folin, O., and Wu, H., *J. Biol. Chem.*, 41, 367 (1920).

The results of a typical experiment are given in the table. Examination of the data shows that oral administration of glucose to fasting rabbits, in addition to producing marked hyperglycemia, is accompanied by an appreciable lowering of the pantothenic acid content of the blood.

Rabbit No.	Treatment	Blood sugar, mg. per 100 cc.				Blood pantothenic acid, micrograms per 100 cc.			
		0 min.	30 min.	60 min.	90 min.	0 min.	30 min.	60 min.	90 min.
I	Untreated	116	112	118	110	21	20	20	20
II	10 cc. H_2O	120	105	113	105	34	35	34	31
III	5 gm. glucose	85	206	203	140	34	28	25	23
IV	5 " "	92	252	248	170	34	30	28	27

Changes in the level of blood pantothenic acid amounted to 3 to 9 per cent for control rabbits receiving water, and 0 to 5 per cent for untreated controls. These fall within the limits of the method. In contrast, decreases of 20 to 30 per cent in the level of blood pantothenic acid have consistently been obtained following the administration of 5 to 10 gm. of glucose. This is interpreted as additional evidence that pantothenic acid participates in glucose utilization.

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CRYSTALLINE MUSCLE PHOSPHORYLASE*

Sirs:

The enzyme which catalyzes the reversible reaction, polysaccharide + $PO_4 \rightleftharpoons$ glucose-1-phosphate, has been prepared in crystalline form from an aqueous extract of rabbit skeletal muscle. The enzyme constitutes about 2 per cent of the total protein of the extract. The method described below is the outcome of numerous experiments on the stability of the enzyme in different salt solutions at different pH and with and without addition of reducing agents.

All operations are carried out in the cold room. The extract is filtered until clear and dialyzed for 3 hours against running water. Inactive, water-insoluble globulins are removed by adjusting the pH to 6 and filtration. The phosphorylase is then precipitated in 1 per cent glycerophosphate and 1.68 M $(NH_4)_2SO_4$ at pH 7. After standing 12 hours, there is very little enzyme left in the supernatant. The precipitate is collected by centrifugation and dissolved in 1 per cent glycerophosphate and 0.03 M cysteine at pH 7. After dialysis against four or five changes of 0.5 per cent glycerophosphate and 0.004 M cysteine buffer solution at pH 7 and 0° crystals appear. The crystals dissolve in the more concentrated buffer solution at room temperature and recrystallize rapidly on cooling to 0°. The crystals, when first formed, appear as fine, slender needles, but upon standing they increase considerably in size and form long rodlets with sharp edges and blunt ends, visible with low magnification.

The table illustrates the degree of purification achieved by successive recrystallizations. Enzyme activity, expressed in units per mg. of protein, was determined by a method described previously.[1] It may be seen that the activity in the mother liquor approaches more and more that of the dissolved crystals, indicat-

* Supported by a research grant from the Rockefeller Foundation.

[1] Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, 135, 733 (1940).

ing that the mother liquor finally contains only dissolved enzyme protein. This is also indicated by the fact that the third crop of crystals contained 83 per cent of the total protein and 87 per cent of the total activity.

Successive Crystallizations of Muscle Phosphorylase

Crystal-lization	Units per mg. protein		Per cent of total protein in crystals	Per cent of total activity in crystals	Per cent activity without added adenylic acid	
	Crystals	Mother liquor			Crystals	Mother liquor
1*	2390	490	19	54	42	17
2	3050	1570	69	81	63	28
3	3240	2640	83	87	77	47

* The activity in the ammonium sulfate precipitate was 840 units per mg. of protein.

The enzyme crystallizes as an adenylic acid complex. The mother liquor of the first crystallization shows little activity without addition of adenylic acid, while the crystals show up to 77 per cent of full activity when tested without addition of adenylic acid. Furthermore, it has not been possible to crystallize enzyme solutions which are inactive without addition of adenylic acid. As shown previously[1] for enzyme preparations with an activity of 800 to 1200 units per mg. of protein, the crystalline enzyme is inactive in the direction to the left without addition of a trace of glycogen.

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THE STRUCTURE OF SPHINGOSINE*

Sirs:

Levene and West[1] have suggested that sphingosine is 1-amino-2,3-dihydroxyheptadecene-4. Klenk and Diebold,[2] as a result of later studies, revised this formula to 1,2-dihydroxy-3-aminooctadecene-4. The data of the latter authors establish conclusively that sphingosine has a C_{18} chain, a double bond between carbon atoms 4 and 5, and an amino and two hydroxyl groups on carbon atoms 1, 2, and 3. However, their evidence for the relative positions of the substituents is open to question. Therefore, as an initial step in a study of the biochemistry of the cerebrosides, we are making a further investigation of this problem.

Sphingosine sulfate[2] was benzoylated in aqueous alkali in the presence of ether. The crude N-benzoylsphingosine thus obtained was reduced catalytically with platinum oxide to N-benzoyldihydrosphingosine. The latter compound was converted in excellent yield to tribenzoyldihydrosphingosine (m.p. 144–146°) by benzoylation in pyridine. Tribenzoyldihydrosphingosine was hydrolyzed to N-benzoyldihydrosphingosine with hot alcoholic alkali. The N-benzoyl derivative was not obtained in a crystalline form. Its purity was established, however, by analyses and reconversion to tribenzoyldihydrosphingosine in 90 per cent yield.

N-Benzoyldihydrosphingosine was not oxidized by periodic acid under a variety of conditions. Since periodic acid is a specific and powerful oxidizing agent for 1,2-glycols, this result argues strongly against both of the structures proposed for sphingosine, since each of these contains a 1,2-glycol group. N-Benzoyldihydrosphingosine was readily converted into a cyclic acetal by the action of benzaldehyde and zinc chloride. This reaction is characteristic of

* The authors wish to thank The Upjohn Company for a Research Fellowship in support of this work.

[1] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 16, 549 (1913–14).

[2] Klenk, E., and Diebold, W., *Z. physiol. Chem.*, 198, 25 (1931).

either 1,2- or 1,3-glycols. It may be concluded from these reactions that N-benzoyldihydrosphingosine is a 1,3-glycol and that sphingosine, therefore, has the following structure.

$$CH_3(CH_2)_{12}CH{=}CH{-}\underset{\displaystyle OH}{\underset{|}{CH}}{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}\underset{\displaystyle OH}{\underset{|}{CH_2}}$$

We are continuing these studies and are attempting to synthesize sphingosine and dihydrosphingosine.

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MICRODIFFUSION METHODS BASED ON THE BISULFITE REACTION

II. DETERMINATION OF LACTIC ACID BY OXIDATION WITH CERIC SULFATE*

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The Conway microdiffusion unit has proved a useful tool for a variety of biochemical determinations (1). This apparatus, which is simple in design and operation, can be substituted effectively in certain cases for relatively complicated and expensive manometric and distillation equipment. The first paper of the present series described a quantitative microdiffusion method for acetone (2). A similar procedure for the determination of lactic acid is now presented. This method is based on that of Gordon and Quastel (3), who found that lactic acid is oxidized quantitatively to acetaldehyde by ceric sulfate in acid solution at 50°. These investigators employed a current of nitrogen gas to carry the acetaldehyde into bisulfite solution, where the bound aldehyde was determined iodometrically, as in the method of Friedemann, Cotonio, and Shaffer (4).

Johnson and Barger (5) recently have compared the ceric sulfate method of Gordon and Quastel with Edwards' modification (6) of the method of Friedemann, Cotonio, and Shaffer. They find that the two procedures are equally accurate, but that the ceric sulfate method is more complicated and time-consuming.

A lactic acid method which is simpler and more sensitive than these distillation and aeration procedures results if the Conway unit is substituted for the aeration apparatus of Gordon and Quastel. The following are the chief modifications in the latter method. (1) Single lactic acid determinations are performed on

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1 ml. blood samples or on approximately 0.5 gm. of tissue. (2) Zinc hydroxide is used instead of trichloroacetic acid to precipitate blood proteins. The recoveries are low by 5 to 10 per cent if either trichloroacetic or tungstic acid is used.[1] (3) Blood filtrates are freed of sugar by treatment with cupric hydroxide prior to analysis.[2] (4) Determinations are usually conducted at room temperature, instead of at 50°. (5) The acetaldehyde which results from the oxidation of lactic acid passes by simple gaseous diffusion from the outer chamber of the Conway unit into bisulfite solution in the central chamber, thus obviating the need for a current of gas.

Method

Collection of Blood and Preparation of Blood Filtrates—Specimens which are collected in oxalated tubes must be treated with protein- and sugar-precipitating reagents within 10 to 15 minutes of the time the blood is drawn. Glycolysis is prevented if sodium fluoride is used as the anticoagulant. The blood can then stand for several hours in the ice box.

For single determinations, 1 ml. samples of whole blood or plasma are added to 2 ml. of zinc sulfate solution (18 gm. of $ZnSO_4 \cdot 7H_2O$ per liter of N/6 sulfuric acid), and then 1 ml. of approximately N/3 sodium hydroxide is added. The latter solution is adjusted so that 1 ml. just neutralizes 2 ml. of the acidified zinc sulfate solution. After thorough mixing, 1 ml. portions of 10 per cent cupric sulfate solution and 5 per cent calcium hydroxide suspension are added. The mixture is stirred well and centrifuged.[3] The clear, supernatant solution is used for the analysis.

[1] Gordon and Quastel and Johnson and Barger report that trichloroacetic acid does not affect the lactic acid recoveries in the ceric sulfate method.

[2] While the treatment with cupric hydroxide is apparently unnecessary in the case of muscle extracts (5), high lactic acid values result with blood filtrates if sugar is not removed. In trial analyses on a 0.007 per cent lactic acid solution in water containing 60 mg. of glucose per 100 ml., the recoveries were about 10 per cent too high. The starch-iodine color was not permanent at the end-points in these titrations. Friedemann, Cotonio, and Shaffer also found that a bisulfite-binding substance results from the oxidation of glucose.

[3] This technique of precipitating the sugar with cupric hydroxide without preliminary removal of precipitated proteins was used successfully by McCready, Mitchell, and Kirk (7) in their ultramicro lactic acid method.

Preparation of Tissue Extracts—The tissue to be analyzed is frozen *in situ* with liquid air, excised, and pulverized in a chilled crusher, as described by Stone (8). The reagents used in the preceding section for blood are employed in making tissue extracts. About 0.5 to 0.6 gm. of the chilled, powdered tissue is added to a stoppered, tared flask containing 2 ml. of acidified zinc sulfate solution. The flask is restoppered, agitated well, and weighed again. Then 1 ml. portions of sodium hydroxide, cupric sulfate, and calcium hydroxide are added, as in the case of blood. The mixture is centrifuged, and a sample of the supernatant solution is analyzed.

Urine—Urine which contains neither protein nor sugar is analyzed without preliminary treatment. If these substances are present, they are removed in the manner described for blood.

Oxidation and Microdiffusion Procedure—A 3 ml. portion of the solution to be analyzed is pipetted into the outer chamber of a Conway diffusion unit (1). For very high lactic acid levels, smaller samples are used. 1 to 1.5 ml. of approximately 0.25 M sodium bisulfite is placed in the central chamber. The outer rim of the unit is greased with stop-cock lubricant if determinations are to be made at room temperature, or with a vaseline-paraffin mixture (2) if a higher temperature is employed. Approximately 1 ml. of a saturated solution of ceric sulfate in 2 N sulfuric acid is delivered rapidly into the outer chamber with a large tipped pipette, and the unit is immediately sealed with a glass cover-plate. The vessel is rotated to mix the fluids in the outer chamber.

After the unit has stood for at least 5 hours at room temperature, or 2 hours at 50°, the cover-plate is removed and the excess bisulfite is oxidized with 1 N iodine, as described in the acetone microdiffusion method (2). It is not necessary to maintain a low temperature during this oxidation process, as was necessary in the acetone method.[4] If the end-point should be passed, a drop of sodium bisulfite solution is added to remove the excess iodine, and the titration is completed to the starch-iodine end-point. About 0.2 to 0.3 gm. of powdered disodium acid phosphate is added to dissociate the acetaldehyde-bisulfite compound, and the liberated

[4] The acetaldehyde-bisulfite compound has a much smaller dissociation constant than the acetone-bisulfite compound; the former dissociates very slowly in acid solution at 25°, even in the presence of an excess of iodine (4).

bisulfite is titrated with standard 0.003 N iodine. 1 ml. of this solution is equivalent to 0.135 mg. of lactic acid.

In calculation of the lactic acid content of blood, a 3 ml. sample of the blood filtrate is equivalent to 0.5 ml. of whole blood. In the case of tissue, the volume of the final extract is corrected to include the approximate water content of the tissue, assuming that the latter contains 80 per cent water. The lactic acid content of the weighed tissue equals

$$\frac{\text{Lactic acid in aliquot} \times \text{corrected volume of extract}}{\text{Volume of aliquot}}$$

TABLE I

Recovery of Acetaldehyde Obtained upon Oxidation of Lactic Acid

Analyses were performed on 3 ml. aliquots of a solution containing 8.00 mg. of lactic acid per 100 ml. This standard was made up by weight from pure zinc lactate·$3H_2O$.

Time of reaction	Acetaldehyde	
	26°	50°
hrs.	*per cent*	*per cent*
0.5	25	93
0.75		95
1.0	54	95
1.5	77	98
2.0	86	101
2.5		99
3.0	95	
4.0	97	
5.0	99	
6.0	98	

Results

Recovery of Acetaldehyde upon Oxidation of Pure Lactic Acid—In agreement with the observations of Gordon and Quastel, it was found that the oxidation of lactic acid to acetaldehyde at 50° is rapid and quantitative. Table I shows that a period of 2 hours at 50° is sufficient time for complete recovery of the acetaldehyde. At 26° the slower rate of oxidation of lactic acid limits the rate of absorption of acetaldehyde. This fact is indicated by the linear manner in which the recovery of acetaldehyde increases with time

during the first 90 minutes. However, the absorption is complete at the end of 5 hours. It is not advisable to let the units stand for longer periods than are necessary for complete recovery, since there is a steady loss of bisulfite due to diffusion of sulfur dioxide from the central solution into the ceric sulfate solution in the outer chamber, where the gas is oxidized.

Recovery of Lactic Acid from Zinc Lactate Solution after Treatment with Zinc Hydroxide and Cupric Hydroxide—A standard zinc lactate solution containing 48.0 mg. of lactic acid per 100 ml. was treated with protein- and sugar-precipitating reagents, with the proportions specified for blood samples. After centrifugation, analyses were performed on 3 ml. aliquots of the supernatant solution, a temperature of 50° being employed for the oxidation-diffusion process. The recoveries for five determinations were 95, 96, 98, 101, and 102 per cent (average, 98.5). The titrations amounted to about 1.8 ml. of 0.003 N iodine.

It was thought of value to compare these results with those obtainable by a different method for lactic acid determination. Stone's modification (8) of the Friedemann and Graeser method was chosen for this purpose. In this procedure the lactic acid is oxidized by manganese dioxide in boiling solution, and the resulting acetaldehyde is distilled into bisulfite solution. Five determinations were made on samples of the same filtrate used for the microdiffusion analyses of the previous paragraph, and the recoveries were 95, 98, 98, 99, and 100 per cent (average, 98.0).

The two methods apparently have the same degree of accuracy for this level of lactic acid concentration. Stone's distillation procedure is more rapid for single determinations, but the present microdiffusion method makes it possible to run a large number of analyses simultaneously and employs a simple apparatus which can be used for other types of determinations.

Recovery of Lactic Acid Added to Blood—To a sample of blood containing 12.5 mg. of lactic acid per 100 ml. was added an equal volume of a solution containing 35.0 mg. of lactic acid per 100 ml. To a second sample of the same blood was added an equal volume of a solution containing 7.0 mg. of lactic acid per 100 ml. Then protein- and sugar-precipitating reagents were added to each solution, making a 7-fold final dilution of the blood in both cases. After centrifugation five analyses were performed on 3 ml. aliquots

of each of the two supernatant solutions. The recoveries of added lactic acid for the blood filtrate with the higher lactic acid concentration (6.7 mg. per 100 ml.) were 93.5, 94.5, 97.5, 99.0, and 102 per cent (average, 97.5). The recoveries for the second blood filtrate (containing 2.05 mg. of lactic acid per 100 ml.) were 90.0, 95.0, 95.0, 97.5, and 102.5 per cent (average, 96.0). In these two series of determinations, and in all subsequent analyses, the oxidation-diffusion process was conducted at room temperature.

TABLE II

Lactic Acid Determinations in Presence of Different Added Substances

1 ml. portions of solutions containing different organic compounds were added to 6 ml. aliquots of a blood filtrate, each aliquot representing 1 ml. of blood. Duplicate lactic acid determinations were performed on 3 ml. portions of the resulting solutions.

Compound added to blood filtrate	Amount added	"Lactic acid" found
	mg per 100 ml blood	*mg per 100 ml blood*
Water (control)		13.0, 13.3
Urea	100	13.3, 13.5
Uric acid	5	13.5, 14.0
Creatine	10	12.7, 13.0
Pyruvic acid	5	12.7, 13.2
Leucine ..	25	13.5, 13.5
Glutamic acid	25	12.0, 12.5
Alanine	25	14.0, 15.0
Cystine	10	13.5, 15.0
Sodium hydroxybutyrate	25	14.6, 15.3
Acetone	10	17.8, 18.8
Ethyl acetoacetate	25	22.3, 23.0

Possible Interfering Substances in Blood—In order to determine whether any of the compounds commonly present in sugar-free blood filtrates yields volatile bisulfite-binding products upon oxidation with ceric sulfate, a number of these substances were added in pure form to samples of a blood filtrate, and lactic acid determinations were performed on the resulting solutions. The concentrations of the added substances were greater than those normally present in blood filtrates.

That urea, uric acid, creatine, pyruvic acid, leucine, and glutamic

acid do not alter significantly the lactic acid values found for blood filtrates is illustrated in Table II. The slightly elevated values obtained with alanine, cystine, and hydroxybutyric acid are probably not significant. But acetone and acetoacetic acid do produce definitely high "lactic acid" values. Apparently these latter acetone bodies interfere with the determinations when present in considerable amounts. This represents a disadvantage

TABLE III

Blood, Urine, and Tissue Analyses

The values in parentheses indicate the ranges of triplicate or quadruplicate determinations. The lactic acid concentration is expressed as mg. per 100 gm. of tissue for the brain and muscle analyses. The tissues were not frozen in the case of Guinea Pig 2.

Subject	State	Sample	Lactic Acid
			mg. per 100 ml.
Human 1	Resting	Blood*	11.5 (11.0–12.1)
		Plasma	14.4 (13.8–15.0)
		Urine	7.7 (7.5– 7.9)
	After exercise	Blood	38.0 (37.0–38.5)
		Urine	22.1 (20.8–22.4)
Dog 1	Resting	Blood	11.0 (10.7–11.3)
	During ether anesthesia	"	49.5 (48.5–50.0)
" 2	During nembutal anesthesia	Brain tissue	20.5 (19.5–21.0)
" 3	" "	" "	23.5 (23.0–24.5)
Guinea Pig 1	" "	" "	22.0 (21.5–24.0)
		Leg muscle	21.5 (21.0–22.0)
" " 2	" "	Brain tissue	124 (121–127)
		Leg muscle	74 (73–77)

* Analyses of the same blood by Stone's method (8) gave the values 10.7, 11.1, and 11.7 mg. per 100 ml.

compared with the method of Friedemann, Cotonio, and Shaffer, in which preformed acetone and acetone from acetoacetic acid are removed by preliminary boiling and aeration. However, in the case of muscle extracts, no preformed, volatile substances that bind bisulfite are present (5).

Lactic Acid in Blood, Urine, and Tissues—Some applications of the present method to blood, urine, and tissue specimens are illustrated in Table III.

of each of the two supernatant solutions. The recoveries of added lactic acid for the blood filtrate with the higher lactic acid concentration (6.7 mg. per 100 ml.) were 93.5, 94.5, 97.5, 99.0, and 102 per cent (average, 97.5). The recoveries for the second blood filtrate (containing 2.05 mg. of lactic acid per 100 ml.) were 90.0, 95.0, 95.0, 97.5, and 102.5 per cent (average, 96.0). In these two series of determinations, and in all subsequent analyses, the oxidation-diffusion process was conducted at room temperature.

TABLE II

Lactic Acid Determinations in Presence of Different Added Substances

1 ml. portions of solutions containing different organic compounds were added to 6 ml. aliquots of a blood filtrate, each aliquot representing 1 ml. of blood. Duplicate lactic acid determinations were performed on 3 ml. portions of the resulting solutions.

Compound added to blood filtrate	Amount added	"Lactic acid" found
	mg. per 100 ml. blood	*mg per 100 ml. blood*
Water (control)		13.0, 13.3
Urea	100	13.3, 13.5
Uric acid	5	13.5, 14.0
Creatine	10	12.7, 13.0
Pyruvic acid	5	12.7, 13.2
Leucine	25	13.5, 13.5
Glutamic acid	25	12.0, 12.5
Alanine	25	14 0, 15.0
Cystine	10	13.5, 15.0
Sodium hydroxybutyrate	25	14.6, 15.3
Acetone	10	17.8, 18.8
Ethyl acetoacetate	25	22.3, 23 0

Possible Interfering Substances in Blood—In order to determine whether any of the compounds commonly present in sugar-free blood filtrates yields volatile bisulfite-binding products upon oxidation with ceric sulfate, a number of these substances were added in pure form to samples of a blood filtrate, and lactic acid determinations were performed on the resulting solutions. The concentrations of the added substances were greater than those normally present in blood filtrates.

That urea, uric acid, creatine, pyruvic acid, leucine, and glutamic

acid do not alter significantly the lactic acid values found for blood filtrates is illustrated in Table II. The slightly elevated values obtained with alanine, cystine, and hydroxybutyric acid are probably not significant. But acetone and acetoacetic acid do produce definitely high "lactic acid" values. Apparently these latter acetone bodies interfere with the determinations when present in considerable amounts. This represents a disadvantage

TABLE III

Blood, Urine, and Tissue Analyses

The values in parentheses indicate the ranges of triplicate or quadruplicate determinations. The lactic acid concentration is exprcssed as mg. per 100 gm. of tissue for the brain and muscle analyses. Thc tissues were not frozen in the case of Guinea Pig 2.

Subject	State	Sample	Lactic Acid
			mg. per 100 ml.
Human 1	Resting	Blood*	11.5 (11.0–12.1)
		Plasma	14.4 (13.8–15.0)
		Urine	7.7 (7.5– 7.9)
	After exercise	Blood	38.0 (37.0–38.5)
		Urinc	22.1 (20.8–22.4)
Dog 1	Resting	Blood	11.0 (10.7–11.3)
	During ether an-csthesia	"	49.5 (48.5–50.0)
" 2	During nembutal anesthesia	Brain tissue	20.5 (19.5–21.0)
" 3	" "	" "	23.5 (23.0–24.5)
Guinea Pig 1	" "	" "	22.0 (21.5–24.0)
		Leg muscle	21.5 (21.0–22.0)
" " 2	" "	Brain tissue	124 (121–127)
		Leg muscle	74 (73–77)

* Analyses of the same blood by Stone's method (8) gave the values 10.7, 11.1, and 11.7 mg. per 100 ml.

compared with the method of Friedemann, Cotonio, and Shaffer, in which preformed acetone and acetone from acetoacetic acid are removed by preliminary boiling and aeration. However, in the case of muscle extracts, no preformed, volatile substances that bind bisulfite are present (5).

Lactic Acid in Blood, Urine, and Tissues—Some applications of the present method to blood, urine, and tissue specimens are illustrated in Table III.

The lactic acid values are slightly higher in resting human plasma than in whole blood. The concentrations of lactic acid in human blood and urine show marked increases following physical exercise, while dog blood shows a similar rise during ether anesthesia.

The lactic acid values found for brain and muscle agree well with the figures reported by other workers (8–10). The very high levels in the case of Guinea Pig 2, in which the brain and muscle tissues were excised without preliminary freezing, demonstrate the importance of the freezing technique in preventing the accumulation of lactic acid in tissues being prepared for analysis. In these experiments an interval of several minutes elapsed during the weighing of the tissues, before the latter were extracted with acidified zinc sulfate solution. Stone (11) obtained a comparable value, 109 mg. of lactic acid per 100 gm., for mouse brain frozen 15 minutes after decapitation.

In conclusion, the present method appears to be accurate to within about 5 per cent for the range of lactic acid concentrations normally encountered; namely, 10 to 25 mg. per 100 ml. of blood or per 100 gm. of tissue. For higher concentrations of lactic acid, the larger volumes of the iodine titrations increase the accuracy to within about 2 to 3 per cent.

SUMMARY

The Conway microdiffusion unit has been applied to the determination of lactic acid in blood filtrates, tissue extracts, and urine. The lactic acid is oxidized quantitatively by ceric sulfate in the outer chamber of the apparatus, and the resulting acetaldehyde passes by gaseous diffusion into the central chamber, where it is absorbed by sodium bisulfite solution. The diffusion is complete in about 5 hours at 25° or 2 hours at 50°. The bound bisulfite is determined iodometrically.

The author wishes to thank Professor C. G. Johnston, Professor A. H. Smith, Dr. W. E. Stone, and Dr. R. C. Lewis, Jr., for their helpful suggestions and sincere interest in this work.

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MICRODIFFUSION METHODS BASED ON THE BISULFITE REACTION

III. DETERMINATION OF THREONINE BY OXIDATION WITH PERIODATE*

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A quantitative method for threonine, based on the oxidation of this amino acid to acetaldehyde at room temperature by neutral periodate solution, has been described recently by Shinn and Nicolet (1). The acetaldehyde is aerated by a current of carbon dioxide into a series of absorption tubes containing sodium bisulfite solution, and the quantity of bound bisulfite in the latter is determined iodometrically.

Martin and Synge (2), in their recent paper dealing with hydroxyamino acids, could obtain only 70 per cent of the maximum theoretical quantity of acetaldehyde on treatment of *dl*-threonine with neutral periodate solution. These investigators employed a relatively complicated apparatus in which aldehydes were aerated into an absorption tower containing bisulfite solution.

The present author had no difficulty in confirming the finding of Shinn and Nicolet that exactly 1 mole of acetaldehyde results from the oxidation of 1 mole of threonine.

By substituting the Conway microdiffusion unit (3) for the aeration apparatus of Shinn and Nicolet, a procedure for threonine results which is much simpler from a technical standpoint and suitable for the determination of smaller amounts of threonine, of the order of 0.2 to 0.5 mg. A sample of protein hydrolysate is treated with neutral periodate solution in the outer chamber of the Conway unit, and the acetaldehyde resulting from the oxidation of threonine passes by gaseous diffusion into the central

* Aided by a grant from the McGregor Fund.

chamber, where it is absorbed by bisulfite solution. Phosphate buffer is used instead of bicarbonate to maintain a pH of approximately 7.0 during the oxidation process. The sodium arsenite solution used by Shinn and Nicolet to reduce excess periodate is not required in this method.

Method

Preliminary Protein Hydrolysis—An accurately weighed protein sample (usually about 0.5 gm.) is hydrolyzed for at least 24 hours with about 20 ml. of 3 N hydrochloric acid. The hydrolysate is neutralized to a pH of approximately 7.0 with 3 N sodium hydroxide. If hydrochloric acid more concentrated than 5 to 6 N has been used, it is preferable to concentrate the hydrolysate first *in vacuo* to a volume of 5 to 10 ml. in order to remove most of this acid. The neutral solution is made up with water to a suitable volume (100 to 250 ml.), and analyses are performed on aliquots. The preceding operations can be conducted on a much smaller scale if the supply of protein is limited.

Oxidation and Microdiffusion Procedures—1 to 1.5 ml. of 0.25 M sodium bisulfite is pipetted into the central chamber of the diffusion unit. A 2 or 3 ml. aliquot of the solution to be analyzed is pipetted into the outer chamber. 1 ml. of approximately 0.1 M tripotassium phosphate is delivered also into the outer chamber. This solution is adjusted so that 1 ml. will just neutralize 1 ml. of 0.2 M periodic acid (to be added next) to about pH 7.0. The greased glass cover-plate is placed over the unit, leaving a narrow opening. Through this slit 1 ml. of 0.2 M periodic acid[1] is pipetted quickly into the outer chamber. The cover is slid over immediately to seal the vessel completely, and the latter is rotated to mix the solutions in the outer chamber.

After the unit has stood for at least 4 to 5 hours at room temperature, the cover is removed and the excess bisulfite is oxidized with 1 N iodine. Powdered disodium acid phosphate is added to dissociate the acetaldehyde-bisulfite compound, and the liberated bisulfite is titrated with standard 0.005 N iodine. The

[1] This solution is prepared by dissolving crystalline H_5IO_6 in water. It is not feasible to prepare a neutral periodate reagent, since a precipitate results if the periodic acid is mixed directly with the potassium phosphate solution.

titration procedures are described in detail in the previous papers of this series, which deal with microdiffusion methods for acetone (4) and lactic acid (5). 1 ml. of 0.005 N iodine is equivalent to 0.298 mg. of threonine.

Results

Oxidation of Pure Threonine[2]—Table I shows that 4 to 5 hours at room temperature are sufficient for complete absorption of the acetaldehyde resulting from the oxidation of threonine. Despite the difference in the volumes of fluid in the outer chambers, the rates of diffusion are approximately equal in Series A and B.

TABLE I

Recovery of Acetaldehyde Obtained upon Oxidation of Pure Threonine

In Series A, analyses were performed on 3 ml. aliquots of a solution containing 0.102 mg. of threonine per ml. In Series B, analyses were performed on 1 ml. portions of a solution containing 0.305 mg. of threonine per ml.

Time of reaction	Acetaldehyde	
	Series A	Series B
hrs.	*per cent*	*per cent*
1	72	86
1.5	85	
2	93	93
3	96	97
3.5		96.5
4	100	98
5	99	99.5
6	101	99

The initial rate of absorption of the acetaldehyde is considerably more rapid than in the case of the acetaldehyde resulting from the oxidation of lactic acid by ceric sulfate (5). In the latter instance the slower rate of oxidation at room temperature appeared to limit the rate of absorption of the acetaldehyde.

Action of Periodate on Different Amino Acids and on Glucose—In

[2] The threonine used in this study was given to me by Dr. J. M. Orten. This preparation, which was synthesized in the laboratory of C. S. Marvel of the University of Illinois, had 99.0 per cent of the theoretical nitrogen content.

agreement with the observations of Shinn and Nicolet, it was found that amino acids other than threonine do not yield volatile, bisulfite-binding products upon treatment with periodate. Analyses were performed on 1 ml. portions of a solution containing 0.305 mg. of threonine per ml., to which were added 1 mg. samples of different amino acids. Following are the amino acids tested and the corresponding percentage recoveries of threonine (as acetaldehyde): proline 98, histidine 99, cystine 98.5, alanine 101, aspartic acid 99, isoleucine 99, tryptophane 98, and methionine 100.5. These compounds are representative of nearly all of the different structural types of amino acids found in proteins.

A similar observation made by Martin and Synge (2) was that no significant quantity of acetaldehyde resulted when a mixture of fourteen different amino acids (not including threonine) was oxidized with periodate.

In view of the fact that some proteins contain carbohydrate groups, it appeared worth while to confirm the finding of Shinn and Nicolet that glucose does not interfere with the threonine determination. 1 mg. of glucose was added to a 1 ml. aliquot of standard solution containing 0.305 mg. of threonine per ml. Upon analysis of this sample, 99 per cent of the threonine was recovered (as acetaldehyde).

Threonine Content of Proteins—Several purified proteins[3] were analyzed for threonine. The results are summarized in Table II. The casein and gliadin were samples prepared by the late T. B. Osborne. The lactoglobulin was recrystallized three times and dried with alcohol and ether. The chimpanzee hair samples were mixtures from both adult and immature animals. The hair was washed with cold soap solution, extracted for 36 hours at room temperature, first with alcohol and then with ether, and dried in air.

The proteins were hydrolyzed for 26 to 28 hours, 3 N hydrochloric acid being used for the casein and lactoglobulin, 5 N hydrochloric acid for the gliadin, and 8 N hydrochloric acid for the keratin (hair) samples.

Table II shows that the average threonine values agree to within

[3] These proteins were kindly supplied by Professor H. B. Lewis of the Department of Biological Chemistry of the University of Michigan. The lactoglobulin was prepared by Dr. Lila Miller, also of the University of Michigan.

3 to 4 per cent for duplicate protein hydrolysates. Repeated analyses on samples of the same hydrolysate usually agreed to within 2 to 3 per cent.

The average threonine content found for casein, 4.32 per cent, is somewhat higher than the value 3.5 per cent, reported by Shinn and Nicolet (1) and by Block and Bolling (6). The latter investigators employ lead tetraacetate to oxidize the threonine, and aerate the resulting acetaldehyde into a solution of *p*-hydroxy-

Table II

Threonine Content of Some Proteins and Recovery of Threonine Added to Proteins before and after Hydrolysis

All values are calculated on an ash-free, moisture-free basis. The nitrogen content of the proteins, thus calculated, was as follows: casein 14.75, lactoglobulin 14.35, gliadin 17.58, hair, male chimpanzee, 16.29, and hair, female chimpanzee, 16.38 per cent. The values in parentheses indicate the ranges of triplicate or quadruplicate analyses

Protein	Threonine		
	Found	Added per gm of protein	Recovery
	per cent	*mg.*	*per cent*
Casein	4.40 (4.31–4.46)		
	4.23 (4.10–4.32)	61.7*	97.5 (96–99)
Lactoglobulin	5.25 (5.10–5.33)		
	5.46 (5.36–5.59)	55.0*	103 (101–104)
Gliadin	2.92 (2.88–2.96)	21.2†	98.5 (95–102)
Hair, male chimpanzee	6.57 (6.51–6.60)		
" female "	6.77 (6.73–6.87)		

* Added to a portion of the hydrolyzed protein.

† Added to a separate sample of gliadin before hydrolysis.

diphenyl in sulfuric acid, in which the aldehyde is determined colorimetrically.

The average threonine content of lactoglobulin, 5.35 per cent, is considerably higher than that of casein. The recovery of threonine added to portions of the casein and lactoglobulin hydrolysates is seen to be quantitative.

The relatively low threonine content of gliadin, 2.92 per cent, is significant in relation to the work of Rose (7), who found that

this protein was not as effective as casein in supplying the threonine required by young rats.

That threonine is quite unaffected during prolonged acid hydrolysis is indicated by the quantitative recovery of this amino acid in the case in which it was added to gliadin prior to hydrolysis.

It is interesting that the keratin samples have a higher threonine content than casein, gliadin, or lactoglobulin. The value 6.77 per cent threonine for female chimpanzee hair is not significantly higher than the value 6.57 per cent found for male hair.

SUMMARY

A microdiffusion method for the quantitative determination of threonine in protein hydrolysates is described. A sample of hydrolysate is treated with neutral periodate solution in the outer chamber of a Conway unit, and the acetaldehyde which results from the oxidation of threonine diffuses into bisulfite solution in the central chamber. After 4 to 5 hours, the bisulfite which is bound by the acetaldehyde is determined iodometrically.

The percentages of threonine in casein, gliadin, lactoglobulin, and male and female chimpanzee hair are reported.

The author thanks Professors C. G. Johnston and A. H. Smith for their encouragement and interest in this work, and appreciates also the suggestions made by Professor H. B. Lewis of the University of Michigan relative to the presentation of the experimental data.

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THE DISTRIBUTION OF CALCIUM AND MAGNESIUM BETWEEN THE CELLS AND THE EXTRACELLULAR FLUIDS OF SKELETAL MUSCLE AND LIVER IN DOGS

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Although the distribution of sodium, potassium, and chloride in mammalian tissue is now well established, this cannot be said for calcium and magnesium. From analyses of whole tissues for these elements it may be inferred that the magnesium content of the cells, like that of potassium, is relatively high, while calcium must be present in cells in considerably lower concentration than is magnesium, and may, like sodium, be chiefly a constituent of the extracellular fluids.

The latter inference has been supported by the direct analyses of *Thyone* muscle by Steinbach (1), who found that calcium is present in the cells of this tissue in lower concentration than in the surrounding medium. Scott (2) and Scott and Packer (3) have found by use of the electron microscope that the sum of magnesium and calcium in the cells of striated and of smooth muscle is much greater than in the intercellular spaces, but their work gives no clue as to the distribution of magnesium and calcium individually and their observations are quite compatible with a marked difference in the distribution of these two elements.

This paper presents the results of applying principles now accepted as criteria for the distribution of sodium and potassium to determinations of the distribution of magnesium and calcium in the liver and skeletal muscle of dogs. Data for sodium and potassium are also included.

EXPERIMENTAL

Normal dogs were maintained for 2 weeks in metabolism cages on an alternate diet of meat and Globe dog chow. Before the removal of blood and tissues, the dog was weighed, placed on a dog board, and anesthetized by the intravenous injection of 25 mg. of nembutal per kilo of body weight. After 15 minutes, 60 cc. of blood were taken from the femoral artery, of which 45 cc. were collected under oil and 15 cc. were defibrinated for whole blood analyses. One of the rectus abdominis muscles was then quickly removed for the muscle analyses. All incisions were closed with hemostats. After the removal of the muscle, a portion of one of the lobes of the liver was taken for the liver analyses. The blood sample under oil was centrifuged and the serum removed under oil. The muscle required 5 to 6 minutes for removal, after which it was placed immediately in a glass-stoppered weighing bottle. Shortly afterwards, the muscle was placed on a tile and all visible connective tissue and fat removed. It was then returned to the weighing bottle, minced with scissors, and aliquot samples were weighed for all analyses. The removal of the liver required approximately 3 minutes. It was dropped instantly into a flat, glass-stoppered weighing bottle, which had been weighed previously. The tissue was then minced with scissors and dried to constant weight at 101°. After the dried tissue was extracted for neutral fat (4), it was transferred as quantitatively as possible to a special apparatus (5) and pulverized. The dry, fat-free, pulverized liver tissue was kept in a weighing bottle in a desiccator over sulfuric acid. Aliquot samples of liver tissue were weighed for all analyses. Analyses of muscle, liver, serum, and blood from ten normal dogs were made.

Chemical Methods

The following determinations were made on serum: pH, CO_2, water, protein, chloride, sodium, potassium, calcium, and magnesium; on blood: hematocrit, water, chloride, sodium, potassium, calcium, and magnesium; on wet muscle: fat, water, circulating blood, chloride, sodium, potassium, calcium, and magnesium; and, on liver: fat, water, blood, chloride, sodium, potassium, calcium, and magnesium. All tissue analyses were calculated on a fat-

free, blood-free basis. The neutral fat was determined as described in a former paper (4). The amount of circulating blood was determined by the colorimetric comparison of the amount of hemoglobin in the tissue with that of the whole blood, which was taken as nearly simultaneously as possible with the tissues.

The chemical methods for serum, whole blood, muscle, and liver analyses were the same as those used in previous studies (6, 7, 4).

It is imperative that the ignition of the wet muscle and the dry liver for calcium and magnesium analyses be carried out in platinum dishes.

Calculations

The calculations for the extra- and intracellular phases in skeletal muscle and liver were made by means of the equations presented in former papers, or, briefly, the extracellular phase (F) in gm. per kilo of tissue,

$$(F) = \frac{(\mathrm{Cl})_T \times (\mathrm{H_2O})_s \times 1000}{1.04 \times (\mathrm{Cl})_s}$$

in which the subscripts T and s represent tissue and serum, respectively. From the values for (F), the intracellular phase (C) per kilo was estimated by the equation $(C) = 1000 - (F)$. From the values for (C) the intracellular water $(\mathrm{H_2O})_C$ was estimated by the equation $(\mathrm{H_2O})_C = (C)$ minus solids (S) present per kilo of tissue.

The concentrations of sodium, potassium, and magnesium in the extracellular fluid were estimated from their concentrations in serum water by means of factors determined experimentally by Hastings *et al.* (8) and Greene *et al.* (9). The factor for sodium and potassium used was 0.95 and for magnesium 0.77. The concentration of calcium ions in the extracellular fluid was calculated nomographically by the McLean and Hastings equation (10) from total calcium and total serum protein, with a value of 1.97 for $\mathrm{pK_{CaProt}}$, this being the average of values calculated by these authors from data obtained by diffusion methods.

From the amount of estimated phases per kilo of tissue and the calculated concentration of the basic constituents in the extracellular fluids, the concentration of the individual bases in the intracellular phase can be derived. For example, (1) extracellular

tissue magnesium equals the product of the volume of extracellular water and the concentration of the magnesium in extracellular water; (2) intracellular magnesium equals the difference between total tissue magnesium and the extracellular magnesium; (3) the concentration of magnesium per kilo of tissue cells equals the intracellular magnesium divided by the intracellular phase volume; (4) the concentration of magnesium in the intracellular water

TABLE I

Analyses of Blood, Muscle, and Liver of Representative Normal Animals

The values are given for fat-free, blood-free tissue.

	pH	CO_2	H_2O	Cl	Na	K	Ca	Mg	Protein	(*F*)
Dog C_6, weight 25 kilos										
		mM per l.	*gm. per kg.*	*m.eq. per kg.*	*m.eq. per kg.*	*m.eq. per kg.*	*m.eq. per kg.*	*m.eq. per kg.*	*gm. per kg.*	*gm. per kg.*
Serum	7.40	22.12	924.1	107.6	138.5	3.57	4.74	1.08	53.6	
	Gm. per cc.	Hematocrit								
Blood	1.055	50.0	788.0	87.3	119.2	7.24	2.40	2.58		
Muscle			774.0	19.95	30.0	98.5	1.90	16.20		164
Liver			720.0	33.10	38.45	80.9	1.76	18.88		273
Dog C_8, weight 15.4 kilos										
	pH	CO_2								
Serum	7.42	24.77	930.7	109.6	140.5	4.06	4.44	1.80	53.5	
	Gm. per cc.	Hematocrit								
Blood	1.055	43.9	806.2	89.7	121.3	4.82	2.65	2.47		
Muscle			768.0	18.68	26.04	99.0	1.89	17.84		149
Liver			738.0	31.10	31.50	82.5	1.54	12.58		254

equals the intracellular magnesium divided by the volume of intracellular water.

Results

In order to conserve space, the data in full will not be given. In Table I are presented detailed analytical data for two representative experiments from the ten carried out. All of the tissue values are corrected for free, neutral fat and circulating blood.

In Table II are presented the average analytical and derived data with standard deviations for the ten dogs for the distribution of potassium, magnesium, calcium, and sodium in serum, fluids, skeletal muscle, and liver. The analytical data are expressed in

TABLE II

Distribution of Potassium, Magnesium, Calcium, and Sodium between Blood Plasma and Tissue Phases

The values are given in milliequivalents for fat-free, blood-free tissue.

		Potassium	Magnesium	Calcium	Sodium
Serum	Per kilo	4.09	1.73	4.59	138.3
	σ	0.19	0.24	0.35	2.0
	Per kilo H_2O	4.41	1.87	4.96	149.3
	σ	0.24	0.20	0.46	2.5
	Per kilo interstitial fluid	4.19	1.44	3.26	141.7
	σ	0.18	0.20	0.30	2.4
Muscle	Per kilo	97.1	18.11	1.63	23.07
	σ	7.5	1.87	0.35	4.20
	In (*F*)	0.54	0.21	0.45	18.00
	σ	0.15	0.05	0.16	4.30
	In (*C*)	96.6	17.90	1.18	5.07
	σ	7.5	1.37	0.25	1.27
	Per kilo (*C*)	110.5	20.76	1.38	5.80
	σ	7.0	2.00	0.36	1.60
	Per kilo $(H_2O)_C$	151.0	28.42	1.89	7.95
	σ	9.0	2.76	0.46	2.08
Liver	Per kilo	73.2	14.59	1.65	39.5
	σ	6.3	2.04	0.19	4.9
	In (*F*)	1.22	0.43	0.92	39.0
	σ	0.26	0.08	0.19	4.0
	In (*C*)	74.3	14.16	0.76	0.5
	σ	6.7	2.08	0.22	3.9
	Per kilo (*C*)	101.4	20.02	1.07	1.89
	σ	8.6	2.82	0.32	3.00
	Per kilo $(H_2O)_C$	161.2	31.57	1.69	2.8
	σ	10.4	4.68	0.39	4.0

terms of milliequivalents per kilo of fat-free, blood-free tissue. The calculated concentrations of all cations are given per kilo of tissue cells and also per kilo of intracellular water. It will be noted that potassium represents practically all of the univalent

base of muscle and liver cells, and magnesium practically all of the bivalent base. The calculated average potassium concentration per kilo of intracellular water in skeletal muscle was 151.0 milliequivalents ±9.0 and in liver 161.2 milliequivalents ±10.4. The average magnesium concentration per kilo of intracellular water was 28.42 milliequivalents ±2.76 in muscle and 31.57 milliequivalents ±4.68 in liver.

"Excess" sodium in the intracellular water of skeletal muscle was again found in this series of dogs, with an average value of 7.95 milliequivalents ±2.08. In the intracellular water of liver there was only a negligible amount of "excess" sodium and, generally, there was agreement between the amount of sodium found in the liver and the sodium in the extracellular water as calculated.

The calculated mean value for Ca^{++} concentrations in the serum was 3.26 milliequivalents ± standard deviation 0.30 per kilo of H_2O. After the total average calcium per kilo of tissue (muscle 1.63 milliequivalents ± 0.35 and liver 1.65 milliequivalents ±0.19) was corrected for the average extracellular calcium (muscle 0.45 milliequivalent ±0.16 and liver 0.92 milliequivalent ±0.19), the excess calcium per kilo of muscle and liver amounted to 1.18 milliequivalents ±0.25 and 0.76 milliequivalent ±0.22, respectively. Assuming that this calcium is intracellular, the derived concentrations of calcium per kilo of intracellular water in muscle and liver were 1.89 milliequivalents ±0.46 and 1.69 milliequivalents ±0.39, respectively.

DISCUSSION

Analyses of whole tissues for the total content of the individual cations have been numerous, but studies on the distribution of these cations between the two phases of the tissues are few and incomplete. Heretofore, sodium and potassium concentrations between the two tissue phases have been studied more than the magnesium and calcium concentrations. Harrison, Darrow, and Yannet (11) extended their electrolyte studies to include magnesium, but did not include calcium. Since the present investigation considered the concentration of all of the bases occurring simultaneously in the two phases of liver and skeletal muscle in a group of ten dogs, the mean values from such a group should give the basic electrolyte pattern of these tissue cells.

The accuracy of all the derived estimations presented here depends on the correctness of two assumptions; namely, (1) that the chloride may be considered to be confined to the extracellular water, and (2) that the concentration of the different cations in the extracellular fluid can be estimated from their concentrations in serum water by means of the Donnan factors. The factors for sodium, potassium, and calcium were accepted. There is, however, uncertainty about diffusible magnesium. Greene and Power (12) indicate that 77 per cent of the total magnesium is diffusible in the presence of serum protein. Watchorn and McCance (13) and Greenberg (14) have found that about 80 per cent of the magnesium in serum is diffusible. Recent work (15) indicates that as much as 90 per cent may be filtrable instead of the 77 per cent, which has been used in calculating the results in Table II. By comparing the maximum reported diffusion of magnesium with the minimum, the error introduced into the concentration of magnesium in the tissue cell water by the uncertainty of the amount of magnesium in non-ionized combination with the proteins can be calculated. Such a calculation indicated a difference of 0.07 milliequivalent per kilo of intracellular water in muscle and 0.23 milliequivalent per kilo of intracellular water in liver. Thus it is apparent that the intracellular magnesium concentrations are not influenced importantly by this difference in the factor.

The data have been analyzed to see if all of the sodium and calcium may be regarded as extracellular. On the basis of dividing tissues into two phases, after the total content of sodium and calcium in muscle and liver is corrected for the amount in the extracellular spaces, the remaining amount has to be either intracellular or combined with some substance in the extracellular spaces, perhaps connective tissue fibers or the like. That is, if "excess" sodium is assumed to be in the intracellular water, it would amount to a significant average of 7.95 milliequivalents per kilo of muscle cell water, and an insignificant 2.8 milliequivalents per kilo of liver cell water. Most observers (16, 11) agree that "excess" sodium appears in appreciable quantities in skeletal muscle of dogs. The present value falls within the range of those in the literature.

If the calcium of muscle and liver is attributed entirely to the interstitial fluids, the total content of calcium per kilo of tissue

should indicate the volume of extracellular fluids. Since the estimated concentration of Ca^{++} was 3.26 milliequivalents per kilo of interstitial fluid, the total content of calcium found in skeletal muscle and liver would represent 500 gm. of extracellular fluid per kilo of each of the tissues. This volume of extracellular fluid is

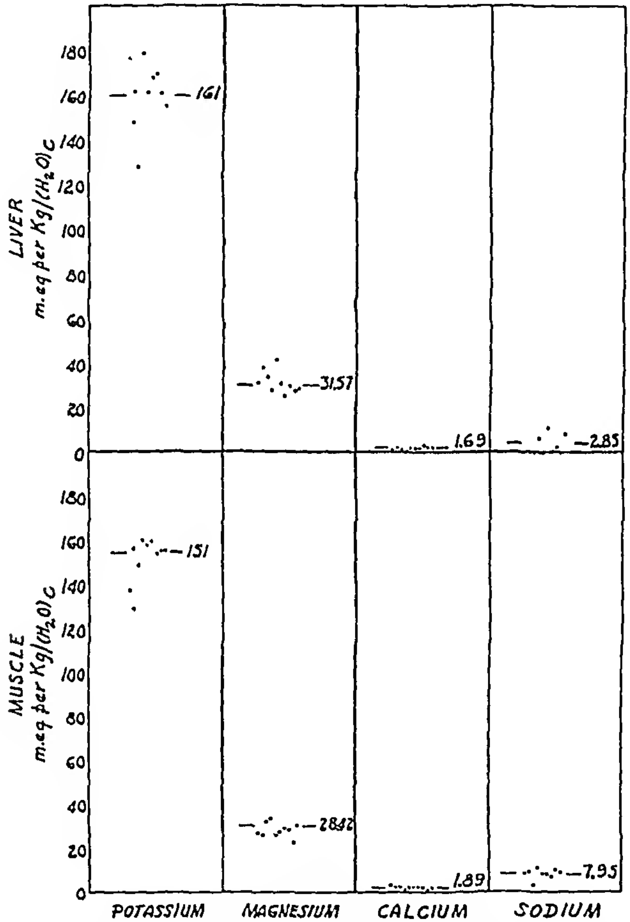

FIG. 1. Individual derivations of the potassium, magnesium, calcium, and sodium contents, expressed in milliequivalents per kilo of intracellular water $(H_2O)_C$ of the skeletal muscle and liver of dogs.

far beyond that calculated by other means. Therefore, the concentration of calcium per kilo of whole tissue indicates that either all of the tissue calcium in the interstitial fluids is not ionized or that there is some calcium in the intracellular phase. Since the excess sodium occurred simultaneously with the excess calcium

in the skeletal muscle tissue, there is a possibility that the "excess" sodium and calcium are combined with the connective tissue fibers in the extracellular phase and are not intracellular.

From Fig. 1 it will be noted that, in both the liver and skeletal muscle, the concentrations of the intracellular bases show considerable scattering, although there is fair average agreement in the two tissues. Since liver and skeletal muscle are two tissues each composed of different types of cells, it is surprising that the basic electrolyte distribution should be so similar.

SUMMARY

By applying methods now accepted as valid for calculating the distribution of sodium and potassium in mammalian tissues to similar calculations for the distribution of magnesium and calcium, we found (1) that the calculated concentration of magnesium in the cells of liver and of striated muscle is high in comparison with that in the blood plasma and the extracellular fluid, being of the order of magnitude of 30 milliequivalents per kilo of intracellular water as compared with 1.5 milliequivalents per kilo of water in the extracellular fluid; and (2) that the calculated concentration of calcium in the cells is lower than that of the extracellular fluid, being of the order of magnitude of 1.8 milliequivalents per kilo of intracellular water as compared with 3.25 milliequivalents per kilo of water in the extracellular fluid. The possibility that the calcium in the tissues is all extracellular has been considered, and the conclusion has been reached that, unless some of it is actually within the cells, a part of it must be assumed to be in an un-ionized combination with some extracellular substance other than the protein of the extracellular fluid, perhaps the connective tissue fibers.

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QUANTITATIVE DROP ANALYSIS

XVI. AN IMPROVED DIFFUSION METHOD FOR TOTAL NITROGEN*

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Although several methods for the determination of 1 to 10 or 20 γ of nitrogen in organic combination have been described, all of them involve technical difficulties, lack of simplicity, or lack of accuracy. Distillation (1), photometric determination of ammonia in the digest (2), and photometric determination of the ammonia after diffusion (3) have been described. In addition, several diffusion-titration methods have been described, the general principle used being that of Conway and Byrne (4). The diffusion method of Kirk and Bentley (5) suffers from the necessity of a transfer of digest, and special precautions relative to manipulation to prevent loss of ammonia. The method of Borsook and Dubnoff (6) requires a glass electrode and other accessories. The procedure of Needham and Boell (7), though apparently simple, is very slow and relatively inaccurate. To combine the advantages of technical simplicity, low cost, and rapid, accurate determination, the procedure described in this paper was developed.

EXPERIMENTAL

Solutions—

Digestion mixture. A good grade of sulfuric acid was diluted with an equal volume of water, saturated with potassium sulfate, and made 0.1 per cent in copper selenite. The latter may be made readily by mixing a concentrated copper sulfate solution with sodium selenite and collecting the precipitate.

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Sodium hydroxide solution, made by diluting saturated NaOH with an equal volume of water.

Standard sodium hydroxide solution, approximately 0.02 to 0.025 N.

Sulfuric acid solution, approximately 0.025 N, containing methyl red indicator. This reagent does not require careful standardization.

Apparatus—The only previously undescribed apparatus used in this procedure was the digestion-diffusion vessel shown in Fig. 1 and the similar vessel shown in Fig. 2. It consisted of an elongated pear-shaped flask, the small bulb of which was used for digestion. Both the small and large bulbs were flattened on one side, the small bulb and its neck being inclined away from the flat area in such a way that the contents of the small bulb would drain freely into the large one when the vessel was placed on its side. It was

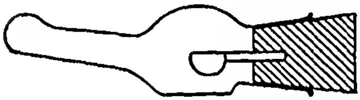

FIG. 1

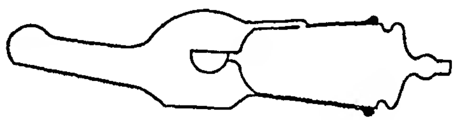

FIG. 2

FIG. 1. Simple digestion-diffusion vessels; one-third actual size.

FIG. 2. Glass-stoppered vacuum digestion-diffusion vessel; one-third actual size.

closed with a rubber stopper carrying a glass cup on a rod handle. The capacity of the cup was between 0.1 and 0.2 ml. The stopper was previously boiled in sodium hydroxide solution and thoroughly rinsed in distilled water.

The second type of vessel shown in Fig. 2 was essentially the same as the first, except that it was sealed by means of a ground glass joint, the female of which was grooved through its lower half, and the hollow plug was drilled to connect with the groove, thus serving as a stop-cock when the plug was rotated. By means of this arrangement the vessel could be evacuated and sealed.

The other equipment necessary included suitable arrangements for carrying out digestions and titration. The first of these may be made in various ways, including submicro burners, electric hot-plates, etc., but use of a sand bath placed on an ordinary electric hot-plate was found most convenient. The titration as-

sembly was that previously described as standard in this series (8, 9). Sampling and titration were performed as previously described (9).

Procedure

The sample, containing from 0.5 to 20 γ of nitrogen, was measured and transferred with rinsing into the bottom small bulb of the vessel. To this was added about 0.1 ml. of digestion mixture. This could be added from a 100 λ[1] capillary pipette or from a graduated 1 ml. pipette, but it was necessary to keep the amount reasonably constant in a series of determinations. The vessel was placed in a sand bath which was heated so that the temperature of the bottom was about 300°. At first, the vessel was placed at an angle of about 10–20° from the horizontal with the small bulb barely under the surface of the sand. After all the water was evaporated from the acid mixture, the vessel was placed upright and pushed deeply into the sand, where it remained until the digestion was complete. It was then removed and allowed to cool. The digest was diluted with about 0.5 ml. of water and, after cooling, 0.3 ml. of half saturated sodium hydroxide solution was added as a layer under the digest. This was accomplished by use of a long tipped capillary pipette which had a little vaseline wiped over the tip and was inserted to the bottom of the digest. The liquid did not adhere and no nitrogen was lost. The absorption cup was greased around the rim with a little vaseline. To it was added, with rinsing, a measured amount of about 50 λ[1] of absorption acid containing methyl red. The volume of acid and rinsings could not exceed 0.1 ml. or the material might spill when the cell was rotated later. While the vessel was held by the small end, the large bulb was brushed several times with the flame of a micro burner. The stopper carrying the acid cup was then inserted and the vessel allowed to cool, leaving in it a partial vacuum. After cooling, the digest and base were mixed in the small bulb and the vessel carefully rolled into the horizontal position so as to wet completely the inside walls with the alkaline mixture, neutralizing any acid which might have spattered or condensed on the walls. The diffusion was allowed to take place at

[1] 1 λ = 1 microliter = 0.001 ml.

room temperature, or at 37° in an incubator, for the necessary length of time. Finally, the absorption cup was withdrawn and the acid titrated back in the cup with standard sodium hydroxide by means of a capillary burette in the usual fashion. A canary-yellow color was taken as the end-point. Blanks were run for each series of determinations, the blank titer including in one value the standardization of the acid and all reagent and indicator errors. From this value the titer of the determination was subtracted and the difference multiplied by the normality of the sodium hydroxide solution to obtain the microequivalents of nitrogen.

Diffusion *in vacuo* is more rapid than at atmospheric pressure. In order to make use of this increased speed, the vessel shown in Fig. 2 was constructed. Its use was identical with the description above, except that after all reagents had been added the ground joint was sealed with stop-cock lubricant and turned in the open position. While the vessel was cooling in ice water, it was evacuated with a pump or water aspirator to about 2 cm. of Hg pressure and the stop-cock closed. The diffusion was allowed to proceed at room temperature, or if convenient at a lower temperature down to about 10°. Below this, sodium sulfate separated from the digest and low results were obtained. Raising the temperature of the evacuated vessel slowed the diffusion owing to the increase of vapor pressure.

When enough vessels were available so that the operator was continually busy charging them and titrating them, as many as 50 determinations were possible in a working day, because most of the time the determination was proceeding without attention on the operator's part.

Results

By use of standard solutions of ammonium sulfate, a study was made of the efficiency of diffusion in the two types of vessel. The results are shown in Fig. 3 as graphs of time against undiffused ammonia. It is seen that the rate of diffusion was markedly increased when the cells were evacuated, but that the greatest increase was in the earlier part of the diffusion. Thus it required little more time to diffuse 100 γ than to diffuse 25 γ of nitrogen, but required appreciably more to diffuse 10 γ than to diffuse 5 γ.

Thus the advantage of using evacuation was much greater with large than with small samples, though in all ranges the rate was increased. This graph serves to determine the necessary diffusion time for any given quantity of ammonia expected in the unknown. If, for example, 10 γ of nitrogen are to be diffused, the times for diffusion may be determined by measuring back along the abscissa from the point of complete diffusion to the intersection on the curve of 10 γ on the ordinate, the times found being about 3 hours at atmospheric pressure and 1 hour at 2 cm. of Hg pressure. The times noted on the graph are maximum, since the points chosen for complete diffusion were rather farther to the right than necessary. These diffusion times for amounts up to 10 γ may conveniently be used for smaller amounts, even though the actual

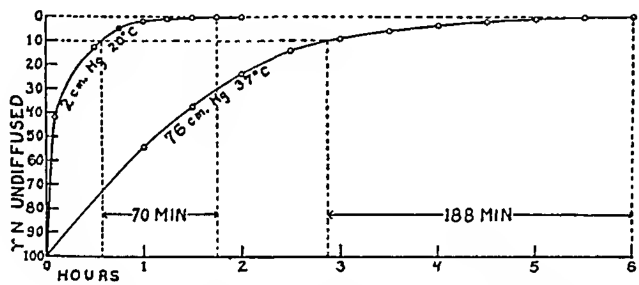

FIG. 3. Time and pressure relations of ammonia diffusion

minimum time is less, since such a choice allows easy standardization of technique. If no information is available as to the amount to be expected, it is wise to allow in the early determinations a relatively long diffusion time, so that no determinations need be wasted.

In Table I are shown the results of diffusing samples of several micrograms of nitrogen from ammonium sulfate solution. This was performed as a test of the diffusion efficiency when the complications introduced by performing digestions were not involved. It is apparent that the diffusion is adequate to recover the ammonia with a precision and accuracy about the same as are obtained with either the macro- or micro-Kjeldahl distillation. The standard errors given refer to deviations from the absolute values which are greater than the deviations from the mean. If the

latter values are used in the same formula, the corresponding values are in no case greater than 0.3 per cent.

Table II gives the results of analysis of varying sized samples of four pure organic compounds. The introduction of the necessary vigorous digestion does not markedly reduce the accuracy of analysis of the contained nitrogen, in amounts down to about 1 γ. Below this value the error is greater.

TABLE I

Diffusion of Ammonia

Ammonium sulfate solution, 0.1 gm. of N per liter.

Sample		Nitrogen found	Recovery	Standard error*	
Volume	Nitrogen				
λ	γ	γ	*per cent*	γ	*per cent*
35.2	3.52	3.48	99.0		
		3.52	100.0		
		3.53	100.2		
		3.51	99.7		
Mean................		3.510	99.7	0.012	0.3
42.1	4.21	4.21	100.0		
		4.20	99.8		
		4.20	99.8		
		4.18	99.3		
Mean................		4.198	99.7	0.0097	0.2

* Standard error $= \pm \sqrt{\Sigma d^2/(n(n-1))}$ where d = deviation from true value, and n is the number of determinations.

It is probable that the difficulties of digestion of the pure compounds listed above and the errors resulting are as great as will be encountered in digestion of biological solutions. However, this matter was not tested previously on the drop scale (5) with protein solutions and the above assumption may not be accepted without experimental evidence. For this reason, the data of Table III were collected. The only criterion of accuracy available in analysis of protein solutions is the comparison with recognized methods. The micro-Kjeldahl analyses used for comparison were made by use of the usual distillation procedure with employment

of the one piece glass distillation apparatus described by Kirk (10). It is noted that the average values obtained by means of the drop scale method were approximately 1.5 per cent lower in the

TABLE II

Analysis of Pure Organic Compounds

Compound	Sample of nitrogen	Nitrogen found*	Standard error†	
	γ	γ	γ	*per cent*
Urea	6.08	6.114 ± 0.046	0.0244	0.4
	6.02	6.078 ± 0.058	0 0335	0.6
p-Aminobenzoic acid	1.47	1.478 ± 0.020	0.0130	0 8
	3.01	3.012 ± 0.018	0.0102	0.3
	4.21	4.206 ± 0.024	0.0134	0.3

Compound	Sample		Nitrogen found	Recovery	Standard error†
	Volume	Nitrogen			
	λ	γ	γ	*per cent*	*per cent*
2,5-Dichloroaniline	14.7	0.588	0.616	104.7	
	30.1	1.204	1.200	99.7	
	14.7	2.940	2.930	99.7	
	30.1	6.020	6.050	100.4	
	42.1	8.420	8.380	99.5	
	100.0	20 00	19.88	99.4	
Mean				100.6	0.9
p-Bromoacetanilide	14.7	0.588	0.597	101.5	
	30.1	1.204	1.180	98.0	
	14.7	2.940	2.985	101.6	
	30.1	6.020	5.960	99.0	
	42.1	8.420	8.380	99.5	
	100.0	20.00	20.00	100.0	
Mean				99.9	0.6

* Mean ± mean deviation from the true value (5 determinations each).
† Calculated as in Table I.

case of albumin solution, and exactly the same in the case of blood serum. The discrepancy in the albumin analyses may have been partly due to the fact that less than 3 γ of nitrogen was present in the drop scale samples, as compared with 0.142 mg. for the micro-

determination. Since the discrepancy may be due to the micro-Kjeldahl analyses as well as to the drop scale analyses, the mean used in calculation of standard error is derived from all analyses.

With the diluted serum, the sample sizes used gave values of 14 γ and 2 mg. respectively for the drop analyses and the micro-analyses. This larger nitrogen content would be expected to yield closer agreement in the two methods. It is not only apparent that correct analyses of protein solutions can be made with the drop scale prodecure, but that in this series of determinations the

TABLE III

Analysis of Protein Solutions

Sample volume	Method	No. of determinations	Nitrogen found*	Standard error†	
		Egg albumin			
			mg. per ml. (γ/λ)	*mg. per ml.*	*per cent*
2.00 ml.	Micro-Kjeldahl	3	0.07097 $\pm$ 0.00076	6.94×10^{-4}	0.97
42.1 λ	Drop scale	4	0.0699 $\pm$ 0.00040	4.41×10^{-4}	0.62
		Diluted blood serum			
2.00 ml.	Micro-Kjeldahl	3	0.9387 $\pm$ 0.00420	3.61×10^{-3}	0.38
14.7 λ	Drop scale	4	0.9387 $\pm$ 0.00175	1.25×10^{-3}	0.13

* Mean $\pm$ mean deviation from the mean.

† Calculated as in Table I except that d = deviation from the mean.

method actually yielded more precise values than did the micro-Kjeldahl analyses.

SUMMARY

A rapid and simple method for the analysis of total nitrogen by diffusion is presented. The accuracy and precision were found to be about equal to those of the micro- and macro-Kjeldahl methods.

No transfers or other disadvantageous techniques were involved in the procedure. It was possible to determine amounts of nitrogen as low as 1 γ with an absolute probable error of not more than about 1 per cent and a precision of about 0.3 per cent. Smaller amounts could be determined with somewhat greater errors.

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THE APPLICATION OF THE SKATOLE COLOR REACTION TO THE DETERMINATION OF FRUCTOSE IN BLOOD*

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This method has been developed as a practicable procedure for estimating the amount of fructose in the dilute tungstic acid filtrate of blood (1). Jordan and Pryde (2) had described a color-producing reaction between fructose and skatole warmed in concentrated hydrochloric acid, which under specified conditions is not affected by the presence of glucose and which they found to be more sensitive than any other reaction used for determining fructose. They suggested that it might be used to measure fructose in blood but gave no procedure. Experiment showed that this reaction is indeed sensitive, but that dilution of the acid by a protein-free blood filtrate unfortunately inhibits it to a marked degree. It was found, however, that the use of ethanol saturated with hydrogen chloride instead of hydrochloric acid causes the reaction to proceed even in the presence of considerable water but also causes the development of an interfering color when heated with skatole in the absence of fructose. This complication was circumvented by adding the skatole after the fructose had been heated with the ethanolic hydrogen chloride and the reaction mixture cooled. Under these conditions the products formed from the fructose react promptly with the skatole, and the

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slow color development which takes place in the absence of fructose in the cold can be minimized by diluting the solution with ethanol after it has reacted for a few minutes. A series of studies on various proportions of ethanolic hydrogen chloride and blood filtrate heated at several temperatures and for different lengths of time showed that 2 parts of the ethanolic hydrogen chloride to 1 part of filtrate heated for 30 minutes at 60° give optimal sensitivity and reproducibility and minimal glucose interference. The detailed procedure developed is given below along with some data demonstrating its degree of accuracy and specificity.

Reagents—

Dilute tungstic acid. 10 Ml. of $\frac{2}{3}$ N sulfuric acid and 10 ml. of 10 per cent sodium tungstate are added to 480 ml. of water with shaking.

Ethanolic hydrogen chloride. Dry hydrogen chloride is bubbled through iced 95 per cent ethanol until it becomes 10.0 to 10.1 N.

Acid ethanol. 4 volumes of hydrochloric acid (sp. gr. 1.1878) are added to 6 volumes of 95 per cent ethanol.

Ethanol; 95 per cent.

Skatole solution. 1 Gm. of recrystallized skatole is dissolved in 100 ml. of 95 per cent ethanol. A few drops of potassium hydroxide are added as a preservative.

Procedure

0.05 Ml. of blood is measured into 5 ml. of the dilute tungstic acid with a capillary pipette graduated to contain, and mixed by a stream of bubbles blown from the tip of the pipette. After 15 minutes the mixture is centrifuged and the supernatant fluid decanted. 2 Ml. of this "filtrate" are transferred to an Evelyn colorimeter tube graduated at 10 and 20 ml. After 4 ml. of the ethanolic hydrogen chloride (caution, keep cold and use a chilled burette to measure) have been added, the tube is covered with a large marble and heated for 30 minutes in a 60° bath. It is then placed in cold water for 3 minutes, after which 0.1 ml. of the skatole solution is added. After another 5 minutes the solution is diluted to 10 ml. with ethanol and between 10 and 15 minutes later the light absorption is measured in an Evelyn colorimeter with a filter having a maximum transmission at 520 mμ. The photometer is set against a blank carried through the procedure with the sample

and identical with it except that it contains distilled water instead of filtrate. If the light absorption is too great for accurate estima-

TABLE I

Interference of Glucose in Determination of Fructose

Fructose present	Glucose present	Fructose found
γ	γ	γ
0	20	0.25
0	20	0.37
0	40	0.25
0	40	0.31
0	80	0.12
0	80	0.44
5	35	5.0
5	35	5.0
10	30	10.25
10	30	10.25
20	20	19.25
20	20	19.75
30	10	29.25
30	10	29.50

TABLE II

Analysis of Blood to Which Known Amounts of Fructose Have Been Added

Blood stored 24 hrs.		Blood stored 72 hrs.	
Fructose added	Fructose found	Fructose added	Fructose found
mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*
0	4	0	1.9
0	4	0	1.9
100	101	0	1.9
100	100	0	1.9
100	101	20	20.3
100	97	20	20.3
100	101	20	23.3
100	97	20	21.5
		20	20.3
		20	22.1

tion, the blank and sample are both diluted to 20 ml. with acid ethanol, the photometer is reset, and a new reading taken.

Table I shows that various amounts of glucose up to 80 γ interfere with the determination to an extent less than the equivalent of 1 γ of fructose.

Table II shows the accuracy with which known amounts of added fructose can be determined in blood. Oxalated dog blood which had been stored on ice was analyzed immediately after the addition of known amounts of fructose.

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A STUDY OF LIPOPROTEINS*

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Lipoproteins, *i.e.* complexes between proteins and lipids, are widely distributed in living matter. They occur in cell nuclei, mitochondria, cell membranes, chloroplasts, in blood, in egg yolk, and in milk. Most of the "heavy" protein fractions isolated from animal tissue, the thromboplastic factor, many viruses, and bacterial antigens have been found to contain lipid-protein complexes. There exists no adequate review of our knowledge of the chemistry of lipoproteins, but some of the pertinent literature will be found in several monographs (1–4) and in a number of recent publications (5–8).

In contrast to the wealth of histological information on the occurrence of lipoproteins in tissues, very little is known of the nature and mode of combination of the lipids in these complexes. One compound of this type, the thromboplastic protein from lungs, has recently been investigated in this laboratory in greater detail (7). It was found to contain a number of different phosphatides (8) and to exhibit complete homogeneity in an electric field (9).

The purpose of the work presented here was to obtain orienting information on two other lipoproteins of biological interest; *viz.*, the phosphatide-vitellin complex occurring in hen's egg yolk (10, 11), which in the following will be designated *lipovitellin*,[1] and *mitochondria* from rabbit liver (12).

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[1] The not infrequent use of the term lecithovitellin for protein preparations which had been freed of phosphatides is unfortunate. It would appear advantageous to reserve such prefixes for the conjugated proteins.

EXPERIMENTAL

Lipovitellin from Egg Yolk

Preparation—The yolks from twelve eggs were carefully washed with water and, after removal of the membranes and chalazae, mixed with an equal volume (220 cc.) of ice-cold saturated sodium chloride solution.[2] The mixture was shaken with 400 cc. of peroxide-free ether and kept in the refrigerator for 4 hours. A small amount of ether extract was removed and the gelatinous aqueous emulsion reextracted with 500 cc. of ether. 4 hours later, following the removal of the ether layer, the mixture was again extracted with 400 cc. of ether. After being cooled overnight, most of the added ether had separated. An additional amount of ether extract was obtained by centrifugation of the aqueous portion. The turbid aqueous solution was dialyzed through cellophane against running tap water for 22 hours. The white protein precipitate was centrifuged off, washed twice with 200 cc. of ice-cold distilled water, and redissolved in 200 cc. of a cold 10 per cent sodium chloride solution. The protein solution was extracted with 200 cc. of ether and after 24 hours in the refrigerator centrifuged in order to obtain complete separation of the ether layer. Dialysis and ether extraction were twice repeated. The final protein precipitate was in addition dialyzed against a large volume of cold distilled water for 17 hours, centrifuged, and washed with 70 cc. of ice-cold distilled water. The protein then was emulsified in 40 cc. of distilled water, the emulsion frozen in a solid CO_2-alcohol mixture, and the water distilled off in a high vacuum. After additional drying over P_2O_5 *in vacuo* the *lipovitellin* was obtained as a white, fluffy powder, weighing 11.4 gm. The protein was only partly soluble in a 10 per cent sodium chloride solution. Analytical data for this preparation (designated Preparation I-Lv) will be found in Table I.[3]

A somewhat different method was used for the isolation of another lipovitellin preparation. In this case, the phosphatides were

[2] All operations were, as far as possible, carried out in an inert gas atmosphere and at a low temperature.

[3] For analysis all preparations were, unless otherwise noted, dried to constant weight *in vacuo* over P_2O_5 at 60° with the exception of the material used for the determinations of the iodine values (13), which was dried at room temperature.

extracted from the emulsion of twelve egg yolks with ether containing 5 per cent of acetone. After repeated dialyses and ether extraction, as described above, the precipitated protein was centrifuged and washed with ice-cold distilled water. A small portion of this preparation was rapidly triturated in 50 cc. of acetone which was kept at −60°. 5 minutes later the mixture was centrifuged in a refrigerated angle centrifuge, and the protein again treated with acetone at −60°. It finally was dried over P_2O_5 *in vacuo*, when 1.52 gm. of a white fluffy powder were obtained (Preparation II-Lv, Table I). The total yield of the lipovitellin Preparation II-Lv can be estimated from subsequent analyses to have been 12.5 gm.

TABLE I

Preparations of Lipovitellin and Vitellin

Preparation No.	N	P	S	Ash
	per cent	*per cent*	*per cent*	*per cent*
I-Lv	13.0	1.5	0.86	3.7
I-A	12.8	1.5	0.83	3.6
I-B	13.0	1.5	0.82	3.8
I-C	12.9	1.5	0.79	3.8
I-D	11.0	1.0	2.8	3.7
I-V	15.5	1.0	1.0	3.4
II-Lv	12.6	1.4	0.88	4.1

The treatment of the lipovitellin preparations with acetone or ether produced practically no change in their composition, as shown in Table I, Preparations I-A (acetone) and I-B (ether). The extraction of lipovitellin with alcohol removed the bulk of the attached lipids and yielded *vitellin* (Table I, Preparation I-V).

Action of Heparin—3.0 gm. of the lipovitellin Preparation I-Lv were suspended in 60 cc. of water and brought into solution by the gradual addition of dilute sodium hydroxide of pH 10. The turbid solution was centrifuged and its volume adjusted to 100 cc. by the addition of dilute sodium hydroxide. The pH of the solution was 10.2.

From 10 cc. of this solution the protein was precipitated by acidification to pH 5.6 by means of dilute acetic acid. It weighed 280 mg. and appeared to be a practically unchanged lipovitellin (Table I, Preparation I-C). To another portion of the solution

(37 cc.) 304 mg. of purified sodium heparin (N 2.3, S 12.0) dissolved in 10 cc. of water (adjusted to pH 10) were added. 15 minutes later the solution was brought to pH 5.2 with dilute acetic acid. The mixture was cooled overnight, and the gelatinous precipitate removed by centrifugation and four times washed with ice-cold 0.6 per cent acetic acid. It then was distributed in a small amount of distilled water and dried in the frozen state in a high vacuum. The almost white fluffy powder weighed 1.14 gm. Its analytical properties are listed in Table I as Preparation I-D. The composition of the two compounds described in this paragraph was not changed by subsequent treatment with ether.

Attempts to remove the lipids attached to the lipovitellin by means of salmine (5) were unsuccessful. Addition of a solution of salmine (pH 10) to the lipovitellin solution mentioned above caused the immediate precipitation of a substance which probably represented a salmine-lipovitellin complex. It contained N 14.5, P 1.3.

Phosphatides in Egg Yolk and Lipovitellin—A portion of the combined ether extracts accumulated in the course of the isolation of lipovitellin Preparation I-Lv, as described above, was evaporated *in vacuo*. The residual oil was emulsified in 80 cc. of physiological saline. The mixture was, after addition of 40 cc. of acetone, cooled and centrifuged. This resulted in the formation of three layers; *viz.*, from top to bottom, of a thin yellow oil, an aqueous layer, and a solid precipitate. The aqueous layer was pipetted off and discarded, and the remainder again treated with 50 cc. of an ice-cold mixture of 2 parts of physiological saline and 1 part of acetone, and centrifuged. Again three layers formed. The aqueous layer was removed, the precipitate treated with 50 cc. of cold acetone, and the mixture centrifuged. This treatment was repeated twice, when no more insoluble oil separated. The solid precipitate was freed of acetone and dissolved in 100 cc. of petroleum ether (b.p. 30–50°). The cerebrosides were removed by freezing in the usual manner. The *phosphatides* (2.3 gm. of a light yellow wax) were precipitated from the final concentrated clear petroleum ether solution by the addition of 4 volumes of acetone. (For the analytical data see Preparation 1 in Table II.[4])

[4] It should be mentioned that similar phosphatide preparations from egg yolk, which will be discussed in a future paper, were found to contain practically no amino acid nitrogen. (Chargaff, E., and Ziff, M., unpublished results.)

In order to obtain information on the quantitative yield of phosphatides which could be isolated by extraction of yolk emulsions with ether, two egg yolks were mixed with 40 cc. of ice-cold saturated sodium chloride solution and extracted with three portions of 160 cc. of ether in the refrigerator. The last extract was practically free of P. The phosphatides isolated, as described in the preceding paragraph, weighed 1.25 gm. and formed an almost white paste. Analytical data will be found under Preparation 2 in Table II.

The *phosphatides retained in the lipovitellin* in a combination strong enough to withstand treatment with ether were extracted with alcohol. Treatment of 3.49 gm. of Preparation I-Lv (Table I) with 100 cc. of absolute alcohol in a Soxhlet apparatus for 14 hours yielded 2.69 gm. of *vitellin* (Preparation I-V in Table I).

TABLE II

Phosphatides in Egg Yolk and Lipovitellin

Preparation No.	P	N	NH_2-N	Iodine value
	per cent	*per cent*	*per cent*	
1	3.8	1.8	0.87	69.6
2	3.6	1.8	0.86	67.9
3	3.8	1.8	0.80	57.3

The *phosphatides* isolated from the alcoholic extract by the customary method weighed 530 mg. (Preparation 3, Table II).

Isolation of Ethanolamine As Diiodosalicylate—It appeared of interest to devise a method permitting the separation and characterization of the bases contained in phosphatides by means of derivatives which could easily be purified and contained no other nitrogen than that of the base (14). The separation procedure was essentially that of Thierfelder and Schulze (15). However, instead of the picrolonate used in that procedure the 3,5-diiodosalicylate of ethanolamine was employed for the isolation of this base. Diiodosalicylic acid appears in this case preferable as isolating agent to picrolonic acid for a variety of reasons; *e.g.*, because of its higher molecular weight and greater solubility in ether and because of the ease with which the purity of the derivatives can be checked by chemical analysis and melting point determination. An example of the separation of choline and

ethanolamine will be given in the next paragraph. For the preparation of *ethanolamine 3,5-diiodosalicylate* 0.203 gm. of ethanolamine was suspended in a solution of 1.4 gm. of 3,5-diiodosalicylic acid in 50 cc. of ether. The salt which began to crystallize almost immediately weighed 1.33 gm. (89 per cent of the theoretical yield). Recrystallization from ligroin-absolute alcohol (4:1) yielded long white needles which melted with decomposition at 199–200° (corrected).

Analysis—$C_2H_7ON \cdot C_7H_4O_3I_2$ (451.0)

Calculated.	C 24.0, H 2.5, N 3.1, I 56.3
Found.	" 24.2, " 2.6, " 3.1, " 56.2

Separation of Bases Contained in Egg Yolk Phosphatides—For hydrolysis, 2.0 gm. of phosphatide Preparation 1, dissolved in a small amount of ether, were added to 40 cc. of 1 N H_2SO_4. The mixture was, after evaporation of the ether, refluxed for 17 hours. The fatty acids were washed with hot dilute sulfuric acid and the combined extracts adjusted to pH 10 by the addition of finely powdered $Ba(OH)_2$. The liquid was neutralized by means of CO_2 and 2 volumes of absolute alcohol were added. The precipitated salts were filtered from the mixture which had been chilled overnight. The filtrate was acidified with HCl and evaporated to dryness *in vacuo*. The dried residue was taken up in absolute alcohol and the solution after centrifugation made up to a volume of 50 cc. It was found by analysis of aliquots of this alcoholic solution that 67 per cent of the amino nitrogen and 95 per cent of the non-amino nitrogen originally present in the phosphatide sample used for hydrolysis had been recovered. The discrepancy between these two values may best be explained by the observation that, because of the presence of unsaturated groups in the phosphatide, too high amino nitrogen values are frequently obtained for the intact phosphatide (16).

The alcoholic solution of the base chlorides (46 cc.) was evaporated to dryness *in vacuo* and the residue dissolved in 1 cc. of water. The solution was very slowly added to freshly prepared calcium oxide in a double thickness extraction thimble which was kept at −20°. The thimble was extracted in a Soxhlet apparatus for 44 hours with 60 cc. of a 1 per cent solution of diiodosalicylic acid in ether. The ethereal extract was, together with the crystalline

material which had deposited during the extraction, evaporated and the residue washed six times with 2 cc. portions of ice-cold ether. It weighed after drying 235.8 mg. (A second ether extraction of the thimble for 24 hours yielded only 2.9 mg. of ether-insoluble material. The combined ether washings of the diiodosalicylate were found to contain 0.12 mg. of N.) Two recrystallizations of the substance from ligroin-absolute alcohol (4:1) yielded 140 mg. of *ethanolamine 3,5-diiodosalicylate* as long white needles melting (with decomposition) at 198–199° (corrected).

Analysis—$C_9H_{11}NO_4I_2$ (451.0).	Calculated.	N 3.1, I 56.3
	Found.	" 3.0, " 56.2

Following the ether extraction the thimble was extracted with absolute alcohol for 7 hours. The extract was concentrated to 20 cc. and 25 cc. of a saturated solution of $HgCl_2$ in alcohol were added. The mixture was cooled, and the white precipitate centrifuged off, and washed with cold alcohol. This material (weight 1.77 gm.) was recrystallized from hot water, when 1.55 gm. of the crystalline *choline chloride-6$HgCl_2$ double salt* (17), m.p. (with decomposition) 249–251° (corrected), were obtained. It was found advantageous to recrystallize this compound from water containing a small amount of $HgCl_2$ (2 to 3 per cent), since otherwise occasionally products of lower $HgCl_2$ content and lower melting point precipitated.

Analysis—$C_5H_{14}NOCl \cdot 6HgCl_2$ (1768.8).	Calculated.	N 0.79
	Found.	" 0.77

The nitrogen balance for this hydrolysis experiment will be found in Table III.

Mitochondria from Rabbit Liver

Preparation—The method of isolation which essentially consisted in extensive gravitational fractionation followed that described by Bensley (12, 18). In a typical experiment, two freshly obtained rabbit livers were perfused through the portal vein with ice-cold physiological saline for about 15 minutes. The tissue then was rapidly frozen by means of solid CO_2 and finely ground

in the frozen and in the thawing state.[5] The very fine pulp was suspended in 250 cc. of saline and subjected to five centrifugations (without decantation) in the angle centrifuge at 1000 R.P.M. for 3 minutes each. The supernatant was three times centrifuged for 5 minutes at 1000 R.P.M. Centrifugation of the supernatant at 4200 R.P.M. for 45 minutes yielded a sediment which was emulsified in 60 cc. of saline. The emulsion was centrifuged at 4200 R.P.M., and the precipitate washed with 60 cc. of saline, and resuspended in 50 cc. of saline. The suspension was left to sediment in a tall cylinder overnight, 45 cc. of the emulsion were drawn off, and the bottom layer again put through several fractional centrifugations. The precipitate obtained by centrifugation at 4900 R.P.M. of the combined supernatants was three times washed with 60 cc. portions of saline, until the trichloroacetic acid reaction of the washings was negative, and three times with water containing a trace of acetic acid. Before the last centrifugation 10 cc. were withdrawn from the suspension which had a total volume of 37 cc., and used in an experiment which will be discussed in the next section of this paper. The *mitochondria* obtained by centrifugation of the remainder were dried *in vacuo* and formed a brown powder weighing 918.1 mg. Analytical data for this material are listed in Table IV as Preparation 1.

TABLE III

Nitrogen Recovery in Hydrolysis of Phosphatide Preparation 1

Fraction	Amino N		Non-amino N	
	Weight	Per cent of phosphatide hydrolyzed	Weight	Per cent of phosphatide hydrolyzed
	mg.		*mg.*	
Phosphatide hydrolyzed	17.4		18.6	
Alcoholic solution of base chlorides	11.7	67	17.7	95
Ethanolamine diiodosalicylate	7.7	44		
Choline chloride-6$HgCl_2$			14.8	80

[5] All operations were carried out at a low temperature (centrifugation and sedimentation in the refrigerator, ice-cold fluids, etc.).

In another similar experiment one rabbit liver yielded 416.1 mg. of *mitochondria* (Preparation 2 in Table IV).

The homogeneity of the preparations was checked by microscopical inspection at various stages of the fractionation. The final preparations, when stained with Altmann's 20 per cent acid fuchsin, were found to consist almost exclusively of red-stained mitochondria together with a few brown patches.

Action of Heparin—The aqueous emulsion of mitochondria Preparation 1 (10 cc.) mentioned in the preceding section was diluted with the same volume of distilled water. To the slightly pink mixture 1.4 cc. of 0.05 N ammonia were added, when it became light brown and viscous. Centrifugation at 4000 R.P.M. for 30 minutes produced practically no sediment. The mixture

TABLE IV

Mitochondria Preparations from Rabbit Liver

Preparation No.*	N	P	S	Ash
	per cent	*per cent*	*per cent*	*per cent*
1	10.6	1.3	0.62	1.6
1a	10.8	1.4	0.87	2.0
1b	8.9	1.0	2.1	1.9
1c	14.2	1.0		
2	11.4	1.1		

* These preparations were dried over P_2O_5 *in vacuo* at room temperature.

was divided into two equal portions. To one portion a solution of 50 mg. of the sodium salt of heparin in 3 cc. of 0.02 N ammonia was added. (Analytical figures for this heparin sample are N 2.1, S 12.4.) Both portions were acidified to pH 4 by the addition of dilute acetic acid. The sample precipitated in the presence of heparin showed slight and gelatinous flocculation which was extremely difficult to centrifuge. Both preparations were centrifuged after being chilled, and the sediments several times washed with water containing a trace of acetic acid. After these were dried over P_2O_5 *in vacuo*, the following preparations were obtained, No. 1a (in the absence of heparin) 127.8 mg., No. 1b (in the presence of heparin) 53.8 mg. of brown powders (analytical data, Table IV).

Examination of Lipids—A sample of mitochondria Preparation

1, ground to a fine powder and weighing 310.4 mg., was extracted in a micro Soxhlet apparatus with a mixture of equal parts of methyl alcohol and chloroform for 23 hours and with ether for 20 hours. The *extracted mitochondria* (Preparation 1c, Table IV) weighed 201.1 mg.

The combined extracts were evaporated *in vacuo* and the residue was treated with 10 cc. of petroleum ether. The chilled mixture was centrifuged and 4 volumes of acetone were added to the concentrated supernatant. The resulting fraction (*Fraction A*) soluble in petroleum ether, insoluble in acetone, (phosphatides?) weighed only 5.0 mg. The petroleum ether-insoluble material was dissolved in warm chloroform. The clear solution obtained after centrifugation was concentrated to a volume of 1 cc. The addition of 5 cc. of acetone produced a precipitate which was centrifuged off, washed with cold acetone, and dried. This substance (*Fraction B*) weighed 20.3 mg. and formed a white powder. Because of the small amount available, only a limited number of analyses could be carried out. The substance was found to contain 46 per cent of cerebrosides (19) and 3.6 per cent of P (20).

The combined mother liquors of Fractions A and B yielded on evaporation *Fraction C*, 52.8 mg. of a light brown fat. This substance was found to contain 3.0 per cent of glycerol (determined as isopropyl iodide), 0.9 per cent of free cholesterol, 5.7 per cent of cholesterol esters (21), and 0.47 per cent of P (20).

DISCUSSION

The lipovitellin preparations described in the present paper were found to contain about 18 per cent of phosphatides which could be removed by extraction of the protein with alcohol. The figures for the phosphatide content of the lipovitellin Preparation I-Lv (Table I), arrived at by comparison of the analytical values obtained for this preparation with those given by the vitellin Preparation I-V (Table I) and the phosphatide Preparation 3 (Table II), were 17.9 per cent on the basis of the phosphorus values, 18.2 per cent on the basis of the nitrogen values, and 16.2 per cent according to the sulfur values. It is probable that the lipoprotein in addition to the phosphatides contained a small amount of other lipids, since the total loss in weight on alcohol

extraction amounted to 23 per cent. As is usually the case with compounds of this type, it was not possible to extract the phosphatides by treatment of the lipovitellin with ether.

The comparison of the composition of the phosphatides which could be removed from the egg yolk by ether extraction with that of the compounds remaining attached to the yolk proteins *revealed only one difference of possible significance: the phos*phatides contained in the lipovitellin had a lower iodine value (compare Table II). In all other respects the composition was very similar. The total phosphatide yield averaged 770 mg. per yolk, of which 144 mg. (18.8 per cent) were present in firm combination with proteins. This is in good agreement with previous results obtained in this laboratory concerning the production of lysophosphatides by the action of snake venoms on egg yolks (22). A discussion of certain points pertinent to the present study, *viz.* the possible biological relationship between the yolk phosphatides and the phosphoprotein vitellin, will be found in the paper following (23).

The analytical figures obtained for the phosphatide-free *vitellin* are in fair agreement with values reported by other investigators (11, 24, 25).

The action of heparin on lipovitellin was studied in view of the recent observation in this laboratory that the phosphatides combined in the thromboplastic protein from lungs were displaced by heparin (26). In the present case too a heparin-protein complex was isolated, but it probably was a compound between heparin and the intact lipovitellin. There was no indication that phosphatides had been split off. The resulting product (Preparation I-D in Table I) had a low ash content, indicating that it was a compound between the protein and the free heparin acid. If, therefore, the computation is based on the formula of free heparin, proposed by Charles and Todd (27), $C_{28}H_{44}O_{38}N_2S_5$ (S 13.6, N 2.4), the following figures for the heparin content of Preparation I-D (Table I) are obtained by comparison with the analytical values given by the lipovitellin Preparation I-Lv (Table I): 15.2 per cent on the basis of the sulfur values, 18.8 per cent on the basis of the nitrogen values. By comparison with the phosphatide-free vitellin Preparation I-V (Table I), on the other hand, the

following heparin percentages are found: 14.3 according to the sulfur values, 34.3 according to the nitrogen values. The agreement between the analytical figures is obviously much better if the compound is formulated as a *heparin-lipovitellin complex.*

Attempts at the preparation of lipid-free vitellin by milder methods than used hitherto, *e.g.* by means of protamines, were not successful. It should be interesting to study the effect of detergent agents on lipovitellin.

The examination of the lipid distribution in *mitochondria* presented in this paper showed that, in confirmation of Bensley's findings (18), phosphatides formed only a small part of the extracted lipids. About 26 per cent of the extract consisted of a fraction which was soluble in chloroform, insoluble in petroleum ether and acetone. The high cerebroside content of this fraction can be reconciled with its high phosphorus value by the assumption that it consisted of cerebrosides and lysophosphatides. Bensley and Hoerr (12) have, in fact, recorded the hemolytic activity of their fractions. The acetone-soluble fraction contained glycerides, cholesterol (almost exclusively in esterified form), and a small amount of phosphatides. The composition of the mitochondria sample examined may tentatively be summarized as follows (in per cent of total dry material): phosphatides 4.0, glycerides 5.4 (as triolein), cholesterol 1.2, cerebrosides 3.3, lysophosphatides (?) 4.3 (as palmityl lysolecithin), extraction residue 72.0. Heparin had a similar effect on mitochondria as on lipovitellin: it combined with the lipoprotein without apparent displacement of the combined lipids.

The composition of both proteins examined remained remarkably unchanged by precipitation from alkaline solutions (Preparation I-C in Table I, Preparation 1a in Table IV).

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SUMMARY

The preparation and some of the properties of two lipoproteins, *viz.* lipovitellin from hen's egg yolks and mitochondria from rabbit liver, are described.

The composition of the phosphatides occurring in the free state in egg yolk and of those combined with the yolk protein vitellin is essentially the same. Lipovitellin contains about 18 per cent of phosphatides (mainly lecithin and cephalin) which are liberated by treatment of the lipoprotein with alcohol. The action of heparin results in the formation of a lipovitellin-heparin complex without displacement of the combined phosphatides.

The examination of the bases contained in egg yolk phosphatides demonstrated the almost exclusive presence of choline and ethanolamine. The latter base was isolated and characterized by means of a new derivative, the 3,5-diiodosalicylate.

The study of the composition of the lipids contained in mitochondria revealed the presence of glycerides, a small amount of phosphatides, cerebrosides, cholesterol esters, and, perhaps, lysophosphatides.

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THE FORMATION OF THE PHOSPHORUS COMPOUNDS IN EGG YOLK*

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The preceding paper (1) presents a study of the lipid-protein complex lipovitellin, contained in hen's egg yolk, and of the phosphatides associated with it. The composition of the phosphatides occurring in the yolk in the free state was shown to be essentially similar to that of the lipids accompanying the vitellin fraction. Whether this similarity extended to the relative rates of synthesis in the organism of the "free" and "bound" lipids remained undecided. Moreover, the occurrence together of phosphoproteins and phospholipids raised the interesting question of a possible metabolic relationship between these two groups of substances. This communication presents an attempt to obtain information on these points with the aid of the radioactive phosphorus isotope.

The origin of the phosphatides deposited by the laying hen in the eggs following the injection of P^{32}_{15} has formed the subject of a careful investigation by Hevesy and Hahn (2). (Compare also (3).) The egg yolk phosphatides apparently are formed in the liver from which they are carried by the plasma to the ovary, where their deposition in the yolk takes place. For a review on the application of radioactive indicators in biology, see Hevesy (4). In the present report the relative rates of formation of the following egg yolk substances are compared: "free" lecithin and cephalin, combined phosphatides (*i.e.* contained in lipovitellin), vitellin, and acid-soluble phosphorus.

The phosphorus compounds isolated from the yolks of eggs

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laid in the course of 8 days following the intramuscular injection of radioactive sodium phosphate were examined individually, as will be described later in this paper. The results obtained with two animals are graphically demonstrated in Figs. 1 and 2. It should be remembered that the yolk is assumed to spend about 240 hours in the ovary, 3 hours in the glandular portion of the oviduct where about half of the egg white is deposited, 1 hour in the isthmus, and 17 hours in the uterus (5).

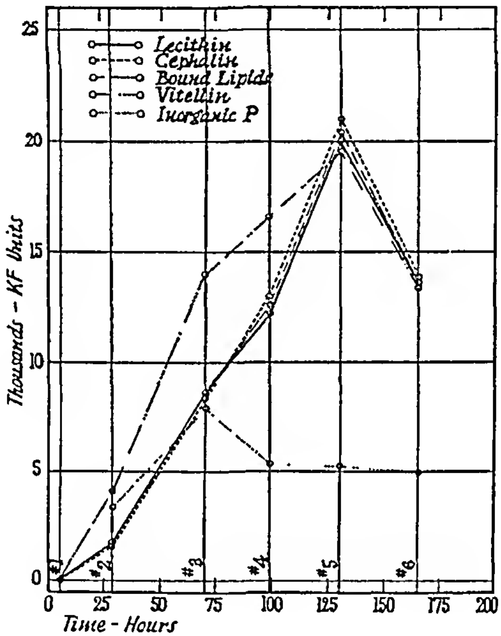

FIG. 1. Concentration of radioactive phosphorus in yolk fractions, Hen I. Ordinate, 10^3 KF units per mg. of P; abscissa, time in hours elapsed between second injection of $Na_2H^*PO_4$ and laying of the eggs (Nos. 1 to 6).

In the egg yolks produced in the first 5 days following the administration of radioactive sodium phosphate the phosphoprotein vitellin exhibited a much higher radioactivity than the phosphatides. This difference disappeared in the later stage of the experiments where the drop in activity experienced with all fractions is, of course, attributable to the progressive dilution of the radioactive phosphorus with inactive phosphorus present in the food and in the body.

The higher activity of vitellin may be explained in a number

of ways. It could, for instance, be assumed that the phosphatides were formed at a slower rate than the vitellin, of which a larger proportion was of more recent origin; or vitellin may possibly be formed in an organ having a higher concentration of the isotopic phosphate than the liver, which presumably is the place of formation of the yolk phosphatides (2). The first assumption appears more plausible at present. It is not unlikely that both the phosphatides and the phosphoprotein are carried to the ovary

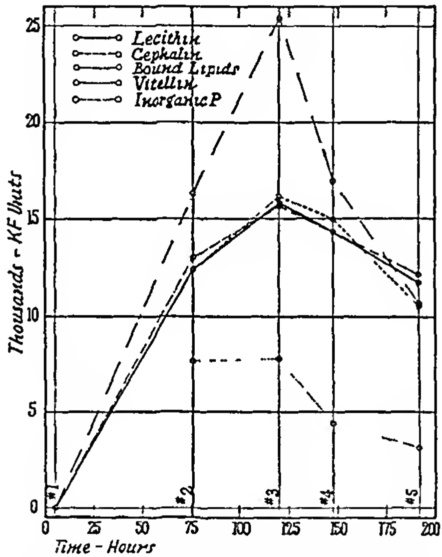

FIG. 2. Concentration of radioactive phosphorus in yolk fractions, Hen II. (For an explanation of the coordinates see Fig. 1.)

by the blood. In the blood of laying hens a considerable increase of the phosphatide level (6, 7) and the presence of vitellin (8) have been demonstrated.

The radioactivities of the various lipid fractions examined were remarkably uniform. Lecithin and cephalin did not differ in the relative speed of their synthesis, as is the case in a number of animal organs (9–12). The absence of differences in radioactivity between the "free" and "combined" phosphatides is significant. The phospholipids occurring in egg yolk in the free

state and those accompanying the vitellin apparently are in equilibrium.

The inorganic phosphorus samples examined (which included the acid-soluble P) uniformly exhibited a fairly low activity. One may consider them a measure of the concentration of inorganic *P prevailing in the plasma or the ovary rather than in the organs where the phospholipids and phosphoproteins of the yolk were synthesized.[1]

The discovery of the presence of the hydroxyamino acid serine in brain phosphatides (13) (compare also (14, 15)) seems to make possible the assumption of a metabolic link between phosphoproteins and phospholipids. It is possible to visualize a mechanism proceeding in the following steps: phosphorylation of the protein at the serine hydroxyl groups; esterification of the phosphoprotein with diglycerides; breakage of the peptide linkages with release of serine phosphatides; decarboxylation of the latter to the corresponding ethanolamine-containing cephalins; etc. The results reported in this paper do not provide sufficient proof of this mechanism; but the larger amount of radioactive phosphorus label found in the vitellin fractions, as compared with the phosphatides, in the earlier stages of these experiments (Eggs 2 and 3 from Hen I; Egg 2 from Hen II) may be considered indicative.

EXPERIMENTAL

Material and Methods

The radioactive phosphorus P^{32}_{15} used was prepared by deuteron bombardment in the cyclotron. It was administered in the form of a neutral aqueous solution of $Na_2H{*}PO_4$.

The radioactivity of the phosphatide and vitellin preparations was measured in the dry state by means of a Geiger-Müller counter, as described previously (16). The radioactivity of the inorganic phosphorus samples examined was measured in solution by an arrangement likewise described in a previous publication (16). The activities are expressed in KF units per mg. of *P.

[1] The asterisk before the symbol for an element indicates an unstable isotope.

Isolation of Phosphorus Compounds

Free Phosphatides—The procedures followed throughout this study were essentially similar to those described in the preceding paper (1). Each yolk was after removal of the membrane suspended in 20 cc. of ice-cold saturated sodium chloride solution. A 2 cc. portion of the mixture was removed for the determination of inorganic P; the rest was three times extracted with 80 cc. portions of ether. It usually required about 12 hours for the layers to separate completely, during which time the mixture was stored in the refrigerator. The ether layers were, whenever necessary, separated by centrifugation. To the combined ether extracts anhydrous Na_2SO_4 and about 10 gm. of finely powdered anhydrous Na_2HPO_4 were added. The mixture was chilled overnight, filtered through celite, and again left in contact with Na_2HPO_4 for several hours. The ethereal filtrate was evaporated to dryness and the residue dissolved in ether. The solution was cleared in the angle centrifuge and concentrated. The addition of 3 volumes of absolute alcohol produced a partly oily precipitate which was washed with alcohol and acetone and purified by reprecipitation with acetone from its solution in ether. The *cephalin* fractions obtained in this manner formed light yellow waxes. The concentrated alcoholic mother liquors yielded on addition of 4 volumes of acetone the *lecithin* fractions, light yellow pastes, which also were purified by reprecipitation.

Vitellin and Combined Phosphatides—The aqueous protein solution remaining after the ether extraction of the yolk emulsion described above was dialyzed through cellophane against running tap water for 20 hours. The precipitated proteins were centrifuged off, washed with distilled water, and suspended in 40 cc. of a mixture of equal parts of absolute alcohol and ether for 5 hours. The protein then was extracted with the same amount of sòlvent for 14 hours and finally with boiling alcohol. It was filtered off, washed with alcohol and ether, and dried *in vacuo*, when the *vitellin* fractions were obtained as white powders. A procedure similar to that described in the preceding paragraph led to the isolation from the alcohol-ether extracts of the *combined phosphatides*. The amounts of cephalin which could be obtained from these lecithin-cephalin mixtures were too small to warrant their separate examination.

Acid-Soluble Phosphorus—To the 2 cc. portion of the yolk suspension in saturated sodium chloride solution, removed before

TABLE I

Radioactive Phosphorus Compounds in Egg Yolks

The values for weight are given in mg. and for P in per cent. The radioactivity figures are given in KF units in 1 mg. of *P.

Hen No.	Egg No.*		Free lecithin	Free cephalin	Combined phosphatides	Vitellin	Inorganic P
I	2	Weight	484.5	28.6	824.5	1,812.3	
	(29)	P	3.2	3.4	3.6	1.1	
		Radioactivity	1,700	1,600	1,700	4,100	3400
	3	Weight	541.8	39.6	948.1	1,811.0	
	(70)	P	3.5	3.2	3.7	1.2	
		Radioactivity	8,600	8,400	8,600	14,000	7900
	4	Weight	524.0	53.0	680.9	1,852.9	
	(98)	P	3.7	3.2	3.8	1.2	
		Radioactivity	12,200	13,000	12,600	16,600	5400
	5	Weight	632.9	37.2	858.5	1,808.0	
	(130)	P	3.5	3.4	3.6	1.1	
		Radioactivity	20,000	21,000	20,400	19,500	5250
	6	Weight	778.2	35.9	696.6	1,743.0	
	(166)	P	3.4	3.3	3.8	1.1	
		Radioactivity	13,600	13,800	13,600	13,400	5000
II	2	Weight	308.9	59.0	792.5	1,846.9	
	(75.5)	P	3.2	3.2	3.5	1.1	
		Radioactivity	12,400	12,400	13,000	16,300	7600
	3	Weight	610.0	80.8	840.7	2,036.5	
	(120)	P	3.4	3.1	3.8	0.9	
		Radioactivity	15,800	16,200	15,700	25,400	7700
	4	Weight	730.0	66.9	670.0	927.1	
	(148)	P	3.3	3.3	3.9	1.0	
		Radioactivity	14,300	15,000	14,300	17,000	4400
	5	Weight	530.6	49.0	759.7	1,978.7	
	(192)	P	3.5	3.4	3.9	1.2	
		Radioactivity	11,700	10,500	12,100	10,600	3100

* The figures in parentheses indicate the time in hours elapsed between the second injection of $Na_2H{*}PO_4$ and the laying of the egg.

the extraction of the phosphatides with ether, 6 cc. of a 10 per cent solution of trichloroacetic acid in water were added. The precipitate was centrifuged and washed with a small amount of water. The combined supernatants were adjusted to a volume

of exactly 10 cc. The radioactivity of the solution was determined as previously described (16), its P content by a colorimetric procedure (17).

Formation of Phosphorus Compounds

Each of two brown Leghorn hens received two intramuscular injections of 1.5 mg. of $Na_2H^*PO_4$ in 1 cc. of water. The second injections were given 18 hours after the first ones. The total amount administered to each animal corresponded to 0.654 mg. of *P with an activity (calculated on the same basis as the values given in Table I) of 66,880,000 KF units.

In the course of the next 8 days Hen I laid six eggs and Hen II five eggs, all of which were examined individually as described in the preceding section. The results obtained are summarized in Table I. In both cases the first eggs were devoid of radioactivity. These eggs (both laid 5 hours after the second injection of sodium phosphate), therefore, are not included in Table I.

The author is highly indebted to Dr. E. O. Lawrence of the Radiation Laboratory of the University of California for the radioactive phosphorus used in these experiments and to Dr. H. H. Darby of this Department for advice and help with the animal experiments. He wishes to express his appreciation to Mr. A. Bendich for experimental assistance.

SUMMARY

The phosphorus compounds (free and combined phosphatides, vitellin, inorganic phosphorus) were isolated from the yolks of eggs laid in the course of 8 days following the intramuscular injection into hens of radioactive sodium phosphate.

The rates of formation of "free" lecithin and cephalin and of the "combined" phosphatides accompanying the vitellin fraction were the same. The phosphoprotein vitellin in the earlier stages of the experiments exhibited considerably higher radioactivity than the phosphatides on a comparable basis.

The possible biological relationship of the phosphoproteins and phospholipids is discussed.

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A NOTE ON THE SEPARATION OF KIDNEY PHOSPHATASES

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Kidney cortex is known to be a rich source of phosphatase. Using sodium β-glycerophosphate as substrate and working over a pH range from 4.0 to 10.0, Bamann and Riedel (1) found that extracts of autolyzed kidney cortex showed activity over the entire pH range studied, with two distinct maxima, one at pH 5.5 to 6.0 and the other at pH 9.0 to 9.5. They concluded that there were two different enzymes present, an acid and an alkaline phosphatase, since it was possible to inactivate either one and still retain the activity of the other (2). A separation of the two enzymes without the inactivation of either one has not yet been described.

During a systematic protein fractionation of beef kidney extracts, we have studied the distribution of phosphatase activity in the various fractions and have found that the acid and alkaline phosphatases can be separated.

EXPERIMENTAL

For determination of phosphatase activity 2.0 cc. of the various enzyme solutions, prepared as described later, were made up to a final volume of 10.0 cc. by the addition of 5.0 cc. of buffered substrate and 3.0 cc. either of water or of various salt solutions. The buffered substrate solutions were prepared by mixing equal volumes of 0.2 M sodium β-glycerophosphate and 0.2 M acetate or veronal buffer (3). Acetate was used in the range from pH 4.0 to 6.0, and veronal in the range from 7.0 to 10.0. The final mixture was incubated at 37° for 2 hours. 5.0 cc. of 10 per cent trichloroacetic acid were then added. The solution was filtered

and the liberated inorganic phosphate determined by the method of Lohmann and Jendrassik (4).

For the determination of protein nitrogen in the enzyme solution 5.0 cc. of 0.1 M metaphosphoric acid were added to 5.0 cc. samples. The resulting precipitate was centrifuged off, washed several times with distilled water, and dissolved by the addition of 10.0 cc. of concentrated sulfuric acid. The nitrogen was determined on aliquots by the Pregl micro-Kjeldahl method.

Fractionation of Kidney Extracts—Fresh beef kidney was freed from blood by perfusion with saline. The cortex was frozen in liquid air and ground to a fine powder in a meat grinder cooled with dry ice. 1 part by weight was added to 5 volumes of 0.5 M NaCl.[1] The mixture was stirred at 3° for 2 hours. The solid particles were centrifuged off and discarded. The clear supernatant was tested for phosphatase activity. The results obtained in a typical experiment are shown in Fig. 1. The activity varies with the pH, showing two distinct peaks similar to those obtained by Bamann and Riedel (1) with extracts of autolyzed tissue.

Fractionation of the extracts was carried out with saturated $(NH_4)_2SO_4$ of pH 6.3, which was slowly dialyzed into the solution in the manner described by McMeekin (5). Four fractions were taken at $(NH_4)_2SO_4$ concentrations of 1.2, 2.0, 2.7, and 3.0 M respectively. The precipitates were separated by centrifugation and tested for phosphatase activity. The solutions for the enzymatic tests were prepared by dissolving the precipitates in a minimum amount of 0.5 M NaCl. These solutions were dialyzed against the same salt solution until ammonia-free.

The first two fractions separated, that is the proteins precipitated up to 2.0 M, were found to be inactive. The next two contained phosphatase, the fraction separating at 2.7 M having both acid and alkaline activity, and that separating at 3.0 M having only acid activity. The two active fractions were combined, dissolved in a minimum of distilled water, and dialyzed against distilled water at 3° until free from $(NH_4)_2SO_4$. A globuin precipitate which was separated during dialysis and the clear

[1] The NaCl solutions throughout were always adjusted to pH 7.0 with $NaHCO_3$.

supernatant solution were both tested for phosphatase activity. The results are represented in Fig. 1.

The phosphatase activity of the supernatant solution was distributed over the whole pH range with a maximum at pH 5.5 and a rapid decrease at more alkaline reactions. The precipitate, on the other hand, showed little activity in the acid range, and a sharp maximum at pH 9.3.

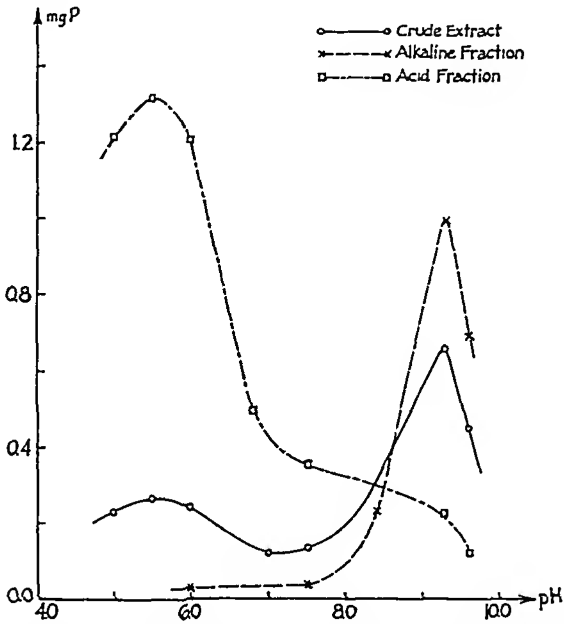

Fig. 1. Distribution of phosphatase in the crude extract and the active fractions. Phosphatase activity is expressed as mg. of P hydrolyzed in 1 hour per mg. of protein nitrogen at 37°.

It would thus appear that the acid and the alkaline phosphatase activities are associated with two different protein fractions, which can be separated by taking advantage of differences in their solubilities. The acid phosphatase is a water-soluble protein. The alkaline phosphatase is a water-insoluble, salt-soluble globulin.

The action of divalent cations on alkaline kidney phosphatase has been studied by several investigators (6–8). Mg and Mn have

TABLE I

Effect of Mn and Zn on Alkaline and on Acid Phosphatase Fractions

Phosphatase activity is expressed as mg. of P hydrolyzed in 1 hour per mg. of protein nitrogen at 37°.

Preparation No.	Metal ion added	Final concentration	P hydrolyzed	Change
		Alkaline fraction		
		M	*mg.*	*per cent*
1	None		0.175	
	Mn	0.001	0.417	+148
		0.0005	0.567	+225
		0.0001	0.685	+290
		0.00005	0.66	+278
		0.00001	0.502	+186
2	None		0.535	
	Mn	0.001	1.23	+130
		0.0005	1.73	+224
		0.0001	2.14	+300
1	None		0.175	
	Zn	0.001	0.035	−80
		0.0005	0.045	−61
		Acid fraction		
1	None		0.532	
	Zn	0.01	0.253	−52.5
		0.005	0.354	−33.5
		0.001	0.454	−15.0
2	None		1.56	
	Zn	0.01	0.725	−53.5
		0.005	1.04	−33.2
		0.001	1.30	−16.4
3	None		1.58	
	Zn	0.01	0.79	−53.0
		0.005	1.10	−30.5
		0.001	1.36	−15.5
1	None		0.532	
	Mn	0.01	0.406	−23.0
		0.001	0.542	+2.0
		0.0001	0.548	+3.0
2	None		1.56	
	Mn	0.01	1.26	−19.0
		0.001	1.58	
		0.0001	1.56	
3	None		1.58	
	Mn	0.01	1.36	−14.0
		0.001	1.57	
		0.0001	1.63	+3.0

been found to activate this enzyme, whereas Zn has a slight inhibitory effect. Little is known about the effect of these cations on the acid enzyme. Mg has no influence on the activity of the acid phosphatase, as shown by Bamann and Riedel (1).

Further evidence for the existence of two enzymes, of which the chemical properties and presumably also the functions in the organism are different, is based on the action of Mn and Zn on the kidney phosphatases.

The results obtained with the two fractions are represented in Table I. Zn has an inhibitory effect on both enzymes, whereas Mn activates the alkaline enzyme with an optimum effect at a total concentration of 0.0001 M. At this concentration Mn has no effect on the acid enzyme. Higher concentrations do, however, cause a slight inhibition.

SUMMARY

The acid and the alkaline phosphatases of kidney cortex extracts have been separated as two distinct protein fractions.

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THE CHEMICAL NATURE OF ACTINOMYCIN, AN ANTIMICROBIAL SUBSTANCE PRODUCED BY ACTINOMYCES ANTIBIOTICUS*

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In the study of the formation by soil microorganisms of antimicrobial substances possessing bacteriostatic and bactericidal properties, a species of *Actinomyces* was isolated and described as *Actinomyces antibioticus* (1). This organism was found (2) to produce a substance, designated as *actinomycin*,[1] which had a marked bacteriostatic effect against all bacteria, actinomycetes, and fungi tested; Gram-positive bacteria were much more sensitive than Gram-negative organisms, with considerable variation within each group. By the use of petroleum ether, it was possible to separate the active substance into two constituents; namely, actinomycin A, orange-red in color, and actinomycin B, colorless; the first was crystallized and studied in detail. This paper deals with the nature and properties of actinomycin A.

The substance was obtained by growing the organism on a medium consisting of 1 per cent tryptone peptone, 0.5 per cent starch, 0.2 per cent K_2HPO_4, 0.2 per cent NaCl, and 0.25 per cent

* Journal Series paper of the New Jersey Agricultural Experiment Station, Rutgers University, Department of Soil Chemistry and Microbiology.

[1] This name is not to be confused with the designation *actinomycetin*, suggested by Welsch (3) for preparations of certain actinomycetes, which have a lytic action upon specific bacteria. Actinomycin is different from actinomycetin in its chemical and biological properties and has now been isolated and crystallized, whereas the latter is merely a designation for a substance which has not been isolated or a process which is not as yet sufficiently understood.

agar in distilled water. It was also formed, in lower concentration, however, on a synthetic medium. On the organic medium, growth of the organism proceeds rapidly at 25–35° and is accompanied by the formation of a soluble dark brown pigment. The surface of the medium becomes rapidly covered with a white aerial mycelium having a faint yellowish green tinge. After 6 to 10 days incubation, the cultures are extracted with ether for 6 to 12 hours; this is repeated three to four times. The combined extracts are evaporated to dryness and extracted with petroleum ether, leaving a residue rich in actinomycin A. For testing purposes, the residue is dissolved in 95 per cent ethanol and the resulting solution is diluted with sterile water. The yield of the total crude active preparation is about 150 mg. per liter of medium and of actinomycin A about 100 mg. Actinomycin B has very little or no bacteriostatic action, but is frequently strongly bactericidal; this property does not seem to be constant.

Chemical Properties of Actinomycin A

The purification of crude actinomycin A was effected by chromatographic adsorption followed by fractionation of the eluate. For this procedure, the orange-brown residue remaining after the petroleum ether extractions was dissolved in benzene, filtered from a small amount of black material, and allowed to pass through a tower packed with aluminum oxide (Brockmann). On washing the tower with large amounts of benzene, a chromatogram slowly developed. The column was then washed with a solution of acetone and benzene (15:85) until the yellow-orange band approached the bottom of the column. The elution of the pigment from the column was accomplished finally by further washing with 30 per cent acetone in benzene until the eluate was faintly yellow in color. The latter eluates were found by assays to contain all the active pigment, whereas all previous eluates, as well as the fractions remaining on the adsorbent, showed no bacteriostatic or bactericidal activity.

Pure actinomycin A was obtained by concentrating the 30 per cent acetone-benzene eluates to dryness, followed by recrystallizing the red solid residue from acetone-ether mixtures or from ethyl acetate. From these solvents, the pigment separates as vermilion-red platelets which melt at 250°, with slow decomposition. The

pigment is readily soluble in chloroform, benzene, and ethanol, moderately in acetone and hot ethyl acetate, and to a slight degree in water and ether. The color of the solid pigment depends on its state of subdivision; when ground very fine, its color is orange-red.

The homogeneity of actinomycin A was established by examination of the optical rotations and of absorption spectra (visible) of fractions obtained by repeated recrystallizations of the pigment from acetone and from ethyl acetate.

Actinomycin is optically active, a solution of 5.00 mg. in 2 cc. of ethanol in a 1 dm. tube having a rotation $-1.60°$; $[\alpha]_D^{25} = -320° \pm 5°$. As shown by the analytical results reported in Table I,

TABLE I

*Chemical Analysis of Actinomycin A**

Carbon†	Hydrogen†	Nitrogen†	$-OCH_3$	$-NCH_3$	$-CCH_3$	$COCH_3$‡
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
59.01	6.81	13.35	0	6.32	7.72	2.85
				6.05	6.19	2.88

* The samples were dried at 100° at 1 mm.

† Average of at least ten analyses on four different specimens.

‡ Alkaline hydrolysis was necessary for the detection of acetyl; acid hydrolysis gave negative results. It has not been established that the volatile acid obtained in these determinations is acetic acid. This fact and the low analytical values need further examination.

it contains carbon, hydrogen, oxygen, and nitrogen. Sulfur, phosphorus, halogens, and metals are absent.

The molecular weight of actinomycin A, as determined by the Rast method, with carbon tetrabromide as the solvent, was found to be around 1000. Cryoscopic measurements in cyclohexanol and in phenol were also made. In the former solvent, the molecular weight is 768 to 780, whereas in the latter the value is 813. Sufficient data are not yet available for assigning to actinomycin A a molecular formula free from reasonable doubt, because of the high molecular weight of the pigment. Table II contains a few of the many possible molecular formulas. The assignment of the correct formula must await careful investigation of degradation experiments.

Actinomycin A exhibits characteristic absorption in the visible

and ultraviolet regions. In ethyl alcohol, it shows strong absorption at 450 mμ ($E_{1\,cm.}^{1\%}$ = 200; Fig. 1) and between 230 and

TABLE II

Possible Molecular Formulae for Actinomycin

Molecular formula	Mol. wt.	C	H	N
		per cent	*per cent*	*per cent*
$C_{41}H_{56}N_8O_{11}$	837.5	58.86	6.71	13.38
$C_{37}H_{50}N_7O_{10}$	752.6	59.09	6.91	13.04
$C_{36}H_{49}N_7O_9 \cdot \frac{1}{2}H_2O$	732.6	59.18	6.91	13.38

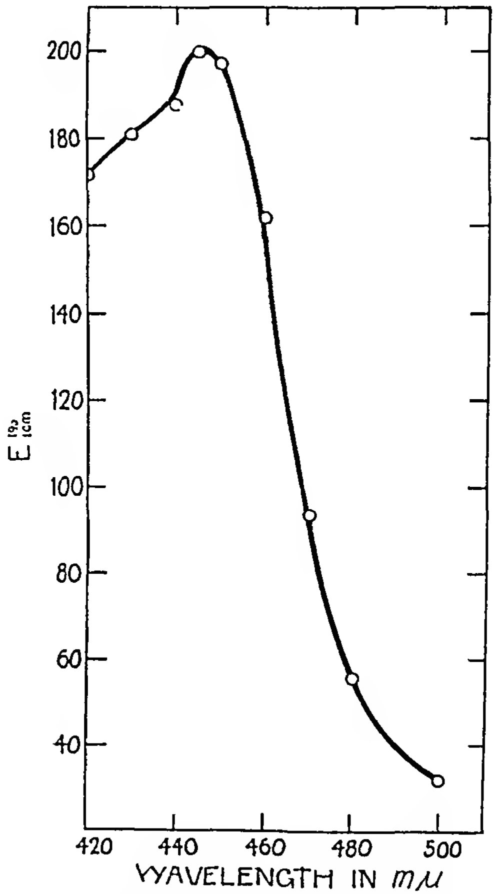

FIG. 1. Absorption spectrum (visible) of actinomycin A, in 95 per cent alcohol.

250 mμ (Fig. 2). Slight shifts of the position of the maximum of the ultraviolet absorption spectrum have been observed in the

case of some samples on standing. Accompanying this shift was a marked increase in the intensity of the absorption. This behavior is indicated in Fig. 2 where $E_{1\,\mathrm{cm.}}^{1\%} = 216$ at 241 mμ after 6 minutes; after 21 hours a constant value $E_{1\,\mathrm{cm.}}^{1\%} = 288$ at 244 mμ is reached. The nature of this change is not understood at this time. It is interesting that no difference in the

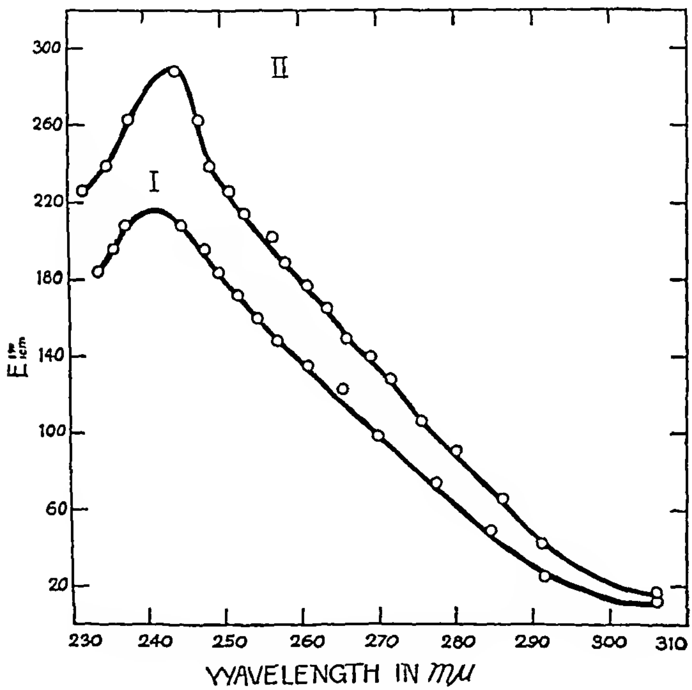

FIG. 2. Ultraviolet absorption spectrum of actinomycin A. Curve I, after 6 minutes; Curve II, after 21 hours (the curve after 27 hours was the same).

optical rotation of freshly prepared solutions and those aged for several days was observed.

Actinomycin A is not soluble in dilute aqueous NaOH or in dilute mineral acids. It is soluble in 10 per cent hydrochloric acid and appears to be regenerated by diluting such solutions with water. With strong alcoholic NaOH (Claisen's alkali), a purple color is formed which, however, rapidly disappears. It does not give a

color with alcoholic ferric chloride. It is readily reduced by sodium hydrosulfite and by stannous chloride, but is unaffected by sodium bisulfite. With sodium hydrosulfite the reduction is characterized by a change in color, red to pale yellow. The color change is reversed by exposing the reduced pigment to air. The same reversibility of color occurs when the pigment is subjected to catalytic hydrogenation in the presence of platinum oxide.

Actinomycin A on reductive acetylation at 10° with acetic anhydride, zinc dust, and pyridine yields a pale yellow com-

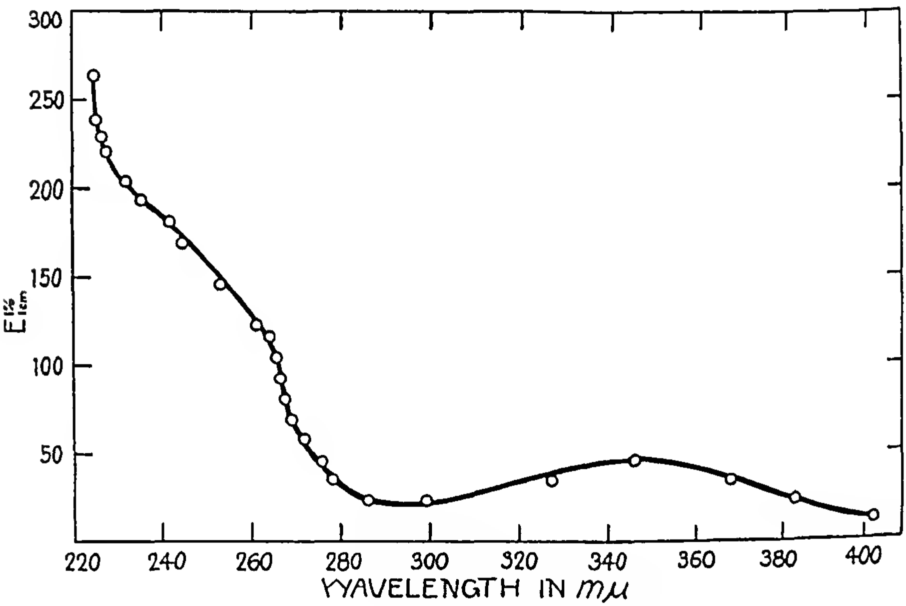

FIG. 3. Absorption spectrum of reduced acetylated actinomycin A

pound which melts at 241° and shows the following analytical values: C, 58.29, 58.52, 58.55; H, 6.33, 6.45, 6.36; N, 12.48, 12.04; acetyl, by acid hydrolysis, 8.73, 8.69; by alkaline hydrolysis, 10.84. Its absorption spectrum (Fig. 3) is decidedly different from that of the pigment (Fig. 2), indicating a fundamental change in the chromophoric group of the parent substance.

Acetylation of the pigment in the absence of zinc dust, at 100° for 3 hours, leads to a new product which has the same color and melting point as the starting material. A mixture of the acetylated compound with actinomycin A, however, melts some-

what lower (246–249°). It showed the following values on analysis: C, 58.27, 58.09; H, 6.56, 6.56; N, 12.36, 12.46; acetyl by alkaline hydrolysis, 10.64.

From the above data, it appears that actinomycin A has one or more functional groups capable of reversible reduction-oxidation (probably quinone in nature) and several others capable of acetylation (probably hydroxyls). The quinone-like structure of the pigment is borne out by its sensitivity to alcoholic NaOH, and to hydrogen peroxide in the presence of sodium carbonate. In the latter instance, the color rapidly disappears and a cleavage seems to occur. In connection with the possibility of a quinone structure for actinomycin A, it should be recalled that Morgan and Cooper (4) demonstrated that quinones have marked bactericidal properties.

Actinomycin in alcohol-water solutions is resistant to the action of heat, being able to withstand boiling for 30 minutes. When such solutions are made acid, however, boiling has a destructive effect upon the activity of the substance, the extent of destruction being directly proportional to the concentration of acid. The effect of alkali, however, is much greater. Dilute alkali changes the color of the substance to light brown, accompanied by a reduction in activity. This can be largely restored when the solution is made neutral again. At a higher alkalinity (0.25 N), especially at boiling temperature, the activity and reversibility are destroyed. Exposure of solutions to light for 1 to 3 months reduces the activity of the pigment very little.

Biological Properties of Actinomycin A

Actinomycin A has been found (2) capable of inhibiting the growth of various bacteria, such as *Sarcina lutea* and *Bacillus subtilis* (Table III), in as low concentrations as 0.01 to 0.1 mg. per liter of medium, which is a dilution of 1:100,000,000 to 1:10,000,000. Assuming that the molecular weight of actinomycin is about 1000, its bacteriostatic action against these bacteria takes place in concentrations of 1×10^{-7} to 1×10^{-8} M concentration. Certain Gram-positive and especially many Gram-negative bacteria, however, are more resistant. The colon-typhoid group is most resistant, 100 to 200 mg. per liter being required to inhibit their growth. Further quantitative data on the effect of actino-

mycin A on various microorganisms are shown in Tables III and IV.

Actinomycin A is highly toxic to animals, 10 γ being sufficient to kill mice weighing 20 gm. in 24 to 48 hours, when injected

TABLE III

Sensitivity of Different Bacteria to Pure Actinomycin A

Growth on incubation of plates, for 48 hours, at 28° or 37°; 0 = none, 1 = trace, 2 = fair, 3 = good.

Concentration per 10 cc. agar	*Bacillus mycoides*	*Bacillus subtilis*	*Sarcina lutea*	*Escherichia coli*	*Aerobacter aerogenes*
γ					
5	0	0	0		
1	0	0	0		
0.5	0	0	0		
0.25	1	1	0		
0.10	3	3	0		
0.05	3	3	3		
mg.					
2				0	1
1				3	3

TABLE IV

Bactericidal Action of Actinomycin A

Bacillus subtilis was used as a test organism.

Concentration of actinomycin	Total viable cells	Spores
mg.		
Start	490,000	66,000
Control*	82,200,000	551,000
0.01	746,000	0†
0.10	284,000	
1.00	110,000	0†

* Incubated for 20 hours at 28°.

† Because of the low dilution of the culture used in plating for spores, it is possible that the actinomycin carried over from the culture into the agar inhibited the developmentof *Bacillus subtilis* on the plate.

intraperitoneally or subcutaneously. It is also highly toxic when administered orally (5).

Actinomycin A may be thus added to the growing list of compounds isolated from microorganisms, which possess bacteriostatic and bactericidal properties. This list now includes penicillin

and gliotoxin, produced by fungi, and pyocyanase, pyocyanine, gramicidin, and tyrocidine, produced by non-spore-forming and spore-forming bacteria. Certain bactericidal preparations produced by different species of actinomycetes have previously been described (6). However, the active principle of these preparations has never been isolated; some of the preparations were claimed to be related to lysozyme, since they were water-soluble and insoluble in ether, alcohol, and other solvents. These preparations also appear to be less toxic than actinomycin A to animals. They differ considerably from actinomycin A in the mechanism of their action upon various specific bacteria. Among 250 or more strains of actinomycetes isolated from various sources and tested so far, none, but *Actinomyces antibioticus*, was found able to produce the typical actinomycin.

The authors wish to express their indebtedness to Mr. H. Boyd Woodruff and Mr. L. G. Ginger for assistance in making some of the preparations used in this work, and to Mr. D. F. Hayman, Mr. W. R. Reiss, Mr. R. N. Boos, Mr. H. S. Clark for the microanalyses.

SUMMARY

A red pigment, designated as actinomycin A, has been isolated in a pure crystalline form from a soil organism, *Actinomyces antibioticus*. Out of 250 strains of actinomycetes tested, no other organism appears to produce this pigment.

Actinomycin contains 59 per cent carbon, 6.8 per cent hydrogen, 13.35 per cent nitrogen, and 20.8 per cent oxygen. It appears to be a polycyclic nitrogen compound.

Actinomycin is optically active, having a molecular weight of about 800. It exhibits characteristic absorption both in the visible and ultraviolet regions. From its behavior towards reducing agents, the pigment appears to have a reversible oxidation-reduction system, apparently of a quinone type.

Actinomycin is an active bacteriostatic and bactericidal (as well as fungistatic and fungicidal) agent, the degree of activity varying with the nature of the organism. It is active in concentrations of 1:100,000,000 against certain Gram-positive bacteria.

Actinomycin is highly toxic to animals.

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A STUDY OF ASCORBIC ACID SYNTHESIS BY ANIMAL TISSUE IN VITRO*

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It is well known that the tissues of albino rats, without an external source of vitamin C, contain appreciable concentrations of ascorbic acid as measured by staining techniques, chemical titration, and biological assay. The relative distribution and absolute concentrations appear to be fairly constant and regulated by synthesis *in vivo*, under most conditions of dietary regimen, but extreme variations in diet and in specific chemical treatment have been reported to induce measurable changes in the distribution and possibly in the total body content of ascorbic acid. Far more striking than the changes in tissue concentration, however, are the changes that can be induced in urinary excretion. When the animals are given a diet of natural foods and fed 20 to 50 mg. per day of such compounds as chloretone, chloral hydrate, or certain barbituric acid derivatives, the urinary excretion of the vitamin commonly rises from approximately 0.2 mg. per day to a range of 10 to 50 mg. per day (1, 2). Lesser degrees of increased excretion are caused by a large number of compounds (3, 4).

A point of special interest has been the correlation between substances that cause an increased excretion of glucuronic acid in some animals and ascorbic acid, or both acids, in others. The preliminary observation that 3-carbon intermediates of carbohydrate metabolism caused an increase in ascorbic acid synthesis (5), although glucose did not, led to a further possible analogy between

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the two acids, since there is also evidence that glucuronic acid does not arise directly from glucose (6, 7). Lipschitz and Bueding have reported studies of *d*-glucuronic acid synthesis by tissue slices (8).

There are a number of reports in the literature which indicate a synthesis of the vitamin by isolated animal tissues (9–15). Some of the reports indicate an appreciable synthesis from one or more of the hexose sugars, particularly mannose, but other workers (16–22) were not able to confirm the work of Guha and others relative to the synthesis from mannose. Von Sztareszky placed special emphasis upon *l*-sorbose (15); Müller (14) and Widenbauer and Koschorreck (19) obtained maximum yields with glucose, and Rudra reported that manganese was an effective agent together with sugars (10). Mitolo (13) reported finding appreciable synthesis by guinea pig tissues. A paper by Mawson (23) shows that the apparent vitamin concentration does not decrease when tissues are incubated for as much as 4 hours; and although he was not concerned with a synthesis of the vitamin, his figures actually indicate a slight increase. So far as we are aware, no one has reported a large number of analyses indicating the degree of variation that occurs in tissue slice experiments.

The apparent acceleration in rate of synthesis that resulted from feeding special chemicals when the animals received a composite diet, such as milk or dog chow, was not observed when a purified diet was fed or when the animals were subjected to inanition. These and other considerations led to the present study of ascorbic acid synthesis by tissue slices, thus permitting more specific control of reaction conditions, especially with reference to (*a*) the substrates from which ascorbic acid might be synthesized and (*b*) the relative activity of different tissues from rats (normal and stimulated), guinea pigs, and humans.

EXPERIMENTAL

The albino rats were raised on a dog chow diet; those reported as "treated" were then transferred to a milk diet plus 20 mg. of chloretone per day for at least 2 weeks in advance of the tests. A few rats received a similar quantity of phenobarbital. The animals were killed by cutting their throats and allowing them to bleed as much as they would. The organ to be used was then re-

moved and sliced. The slices were not washed, because washing removes an appreciable quantity of ascorbic acid. Each slice was weighed on a torsion balance immediately after being cut and this weight was used as a basis for all calculations, the dry weight being considered as 20 per cent of the wet weight. One or more samples were transferred at once to 3 per cent metaphosphoric acid solution for ascorbic acid estimation and adjacent slices were placed in Warburg vessels for the various experiments. All the recorded changes in vitamin concentration are with reference to the concentration found in the slices placed directly in metaphosphoric acid. With liver and brain (cerebrum) no difficulty was experienced in getting successive slices that showed the same content of ascorbic acid. With kidney it was necessary to cut away the outer portion of the cortex before slices were taken, because the outer portion contained as much as 20 per cent less vitamin.

Tissue extracts were prepared by grinding the tissues with twice their weight of Ringer-phosphate solution and centrifuging to give a turbid suspension. Homogenized tissues were also prepared with twice the tissue weight of Ringer-phosphate solution.

The ascorbic acid was determined by the 2,6-dichlorobenzenone-indophenol method essentially as described by Mindlin and Butler (24) and Bessey (25). Most of the determinations were made with 15 second readings on the photoelectric colorimeter and the remainder with 30 second readings. Corrections were applied for the slow reduction and for the turbidity of the solutions.

Attempts to determine the reversibly oxidized form of the vitamin, dehydroascorbic acid, led to a clear demonstration of a possible source of gross error in the procedure that has been commonly used in such analyses. In a number of our experiments with added substrates, reduction by hydrogen sulfide for 1 hour at pH 3.5, followed by removal of the gas with a rapid stream of wet nitrogen, led to the presence of large amounts of reducing substances that could not be differentiated from the vitamin by the standard dye reaction, even with the photoelectric colorimeter. It was then found that treatment of the pure compounds alone (*e.g.* pyruvic acid and glyceraldehyde) with H_2S under the same conditions caused a similar apparent vitamin formation. Direct titration at pH 1 instead of 3.5 caused the 5 second end-point value to be decreased, but the interference was not eliminated. The

reducing action was not destroyed by prolonged aeration, even when the solutions were heated to boiling, but it was destroyed slowly by iodoacetamide or iodoacetate. In view of the results obtained with quinones and with common intermediates of carbohydrate metabolism (Table I), it is evident that values for dehydroascorbic acid obtained by this method can be accepted only with extreme caution, and, further, that the methods for

TABLE I

Reducing Material Formed by Hydrogen Sulfide Treatment

Reaction of product with 2,6-dichlorobenzenoneindophenol within 15 seconds or less; concentration of solutions, 0.001 to 0.0003 M; pH, 3.5; H_2S removed after 2 hours.

Compound*	Approximate mole per cent conversion, calculated as ascorbic acid
Pyruvic acid	35
Glyceraldehyde	40
Dihydroxyacetone	42
2-Methylnaphthoquinone	21
Benzoquinone	25
1,4-Naphthoquinone	46

* The following compounds did not show significant interference: glucose, glucose-6-phosphate, fructose, fructose-1,6-diphosphate, sorbose, xylose, glucuronic acid, levulinic acid, kojic acid, furfural, hexadienal, butyraldehyde, acetone, diacetone alcohol, acetoacetic acid, cyclohexanone, phorone, benzoin, vanillan, piperitone, uric acid, biuret, xanthine, oxamide, propionamide, and 2-methylanthraquinone. Fructose diphosphate caused interference when added to tissues, however. Acetaldehyde, mannosaccharic acid, and 5-ketogluconic acid showed variable interferences, commonly in the range of 0.5 to a few per cent. Crude quinone-rich plant extracts, *e.g.* cascara sagrada, also formed interfering substances.

ascorbic acid analysis which involve clarification with mercuric salts, followed by H_2S treatment, are subject to the same error.

The osazone method of Roe (26), which is often of value for estimating ascorbic acid in the presence of interfering substances, was also tried, but on a micro scale it did not give values that were satisfactory for measuring small differences, particularly in the cases of greatest immediate interest when carbonyl compounds were present as substrates. The substrates tended to precipitate with the vitamin and complicated the rest of the determination.

The ascorbic acid analyses (Tables III and IV) recorded, therefore, represent the material that reduced 2,6-dichlorobenzenoneindophenol within 15 seconds. The general applicability of the method for ascorbic acid, plus the known fact (1, 2) that the treated rats from which tissues were taken were producing and excreting large amounts of ascorbic acid (as shown by biological assay in addition to titration), is sufficient justification, we feel, for calculating and reporting our results as ascorbic acid without reference to the possible quantities of dehydroascorbic acid or interfering substances that might have been present in low concentrations.

In the first experiments slices of kidney and liver from normal and treated rats were placed in Warburg vessels containing Ringer-bicarbonate solution and an atmosphere of 5 per cent CO_2 + 95 per cent O_2. The pH was 7.4. Under such conditions the Q_{O_2} values (c.mm. of O_2 consumed per mg. of dry weight per hour) were large, and in every case a marked loss of vitamin was observed during the experiment. When slices were placed in Ringer-phosphate solution of pH 7.0 with air, the Q_{O_2} values were smaller and the loss of vitamin became much smaller or was changed to a net gain. The Q_{O_2} values obtained under these conditions for liver, kidney, and brain from normal and treated animals are recorded in Table II. Those called "normal" are not normal Q_{O_2} values but Q_{O_2} values for normal tissues under the stated conditions. The values are low, in part because they were calculated on the basis of 20 per cent of the wet weight at the beginning of the experiment. If they were based on the dry weight of the slices remaining at the end of the experiment, they would be considerably higher. Other conditions that contributed to the low values, such as a pH of 7.0 and a large amount of tissue (about 200 mg. of liver and brain, and 100 mg. of kidney), were chosen because of their beneficial effect on the vitamin preservation and for ease of analysis. The values obtained were reproducible as indicated by the standard error in each case. Since the conditions for the normal and treated tissues were the same, the results are strictly comparable for the two groups. Of the Q_{O_2} values, only those for treated brain are significantly different from normal values. The addition of chloretone to the Ringer-phosphate solution bathing the slices lowered the oxygen consumption in all

cases, as had been shown previously for brain (27), but further lowering of the oxygen consumption by the addition of chloretone,

TABLE II

Tissue Slice Values for Q_{O_2} and $Q^{N_2}_{acid}$ under Stated Conditions

Approximately 200 mg. (wet weight) of tissue were used per vessel. The Q_{O_2} values were measured in a phosphate-Ringer's solution of pH 7.0, in an atmosphere of air. The values of $Q^{N_2}_{acid}$ were measured in a bicarbonate-Ringer's solution of pH 7.4, in an atmosphere of 5 per cent CO_2 + 95 per cent N_2. $T = 37°$. 1 hour intervals were used for the calculations.

N. indicates normal tissue; T., treated. ± values represent standard errors.

Tissue	No substrate added				0.01 M glucose as substrate				Intermediates* as substrate			
	No. of experiments	Q_{O_2}	No. of experiments	$Q^{N_2}_{acid}$	No. of experiments	Q_{O_2}	No. of experiments	$Q^{N_2}_{acid}$	No. of experiments	Q_{O_2}	No. of experiments	$Q^{N_2}_{acid}$
Brain. N.	5	2.8 ±0.4	5	1.2 ±0.1	11	2.4 ±0.2	11	3.8 ±0.4	4	3.1 ±0.3	6	2.9 ±0.1
" T.	5	1.8 ±0.2	9	0.9 ±0.1	15	1.9 ±0.1	9	4.3 ±0.1	6	1.9 ±0.2	8	3.0 ±0.2
Liver. N.	6	2.8 ±0.2	15	4.7 ±0.4	4	2.6 ±0.2	4	5.8 ±0.8	4	3.1 ±0.3	4	11.0 ±1.0
" T.	6	3.4 ±0.3	11	1.9 ±0.1	11	3.0 ±0.2	7	2.1 ±0.2	6	3.1 ±0.2	11	5.3 ±0.7
Kidney. N.	4	6.3 ±0.9	7	1.9 ±0.1	10	6.0 ±0.3	5	2.9 ±0.3	4	4.8 ±0.2	4	5.8 ±0.6
" T.	7	5.0 ±0.5	4	1.5 ±0.4	5	5.3 ±0.5	4	3.0 ±0.1	6	5.0 ±0.3	4	7.3 ±0.8

* The term "intermediates" designates a mixture of sodium pyruvate, *dl*-glyceraldehyde, and the potassium salt of hexose diphosphate. The final medium was 0.01 M with respect to each component. We are indebted to Professor H. O. L. Fischer for a generous gift of *dl*-glyceraldehyde.

or by other means such as KCN, did not cause an increase in the vitamin concentration.

The total acid production, $Q^{N_2}_{acid}$, of the different tissues in Ringer-bicarbonate solution with 5 per cent CO_2 + 95 per cent

N_2 is also shown in Table II. The values for liver from treated animals are markedly low. The reason for this difference is apparently not to be ascribed to a difference in fat or moisture content, because the Q_{O_2} values are not lower than those for normal liver. The fact that the livers of treated animals showed markedly lower $Q_{acid}^{N_2}$ values is of interest in relation to other observations on liver function and nerve depressants (28). It will also be noted that $Q_{acid}^{N_2}$ values for brain tissue were markedly increased in both the normal and treated animals by the addition of glucose. Liver and kidney tissues showed greatest acid production when the intermediates[1] (pyruvate + *dl*-glyceraldehyde + hexose diphosphate) were provided as substrate.

The data obtained for changes in the ascorbic acid concentration of brain, liver, and kidney under various conditions are recorded in Table III. In addition to the experiments listed, experiments with other tissues and with the same tissues and other substrates, alone and in various combinations, were carried out. The other substrates tested were glycogen, mannose, sorbose, glucose-6-phosphate, *l*-glycerophosphate, lactic acid, dihydroxyacetone, methylglyoxal, succinic acid, fumaric acid, malic acid, β-hydroxybutyric acid, ethyl acetoacetate, citric acid, acetic acid, creatine, *d*-glucuronic acid, *l*-2-ketogulonic acid,[2] and a number of amino acids, including cystine and cysteine. None of these compounds led to vitamin values exceeding those reported in Table III, and the average values with other substrates were lower than shown for the pyruvate + glyceraldehyde + hexose diphosphate combination. The same was true for combinations of tissues; *e.g.*, liver and kidney. Alternate anaerobic and aerobic periods were

[1] The reasons for choosing these particular intermediates were as follows: It is known that dihydroxyacetone phosphate can condense with a number of aldehydes (29). The product formed by condensation with *l*-glyceraldehyde (*l*-sorbose-1-phosphate) could be converted to ascorbic acid by oxidation at carbon atom 1 and ring closure. Hexose diphosphate was used to supply the dihydroxyacetone phosphate. Pyruvic acid could serve as a possible hydrogen acceptor for the necessary oxidation or as an alternate unit for conjugation with glyceric aldehyde.

[2] The authors are indebted to Dr. Eric Ball for the *l*-2-ketogulonic acid used. Dr. Ball found the compound to be inactive as an antiscorbutic material (30) in guinea pigs, in agreement with independent results obtained in our laboratory at another time.

also tested but they did not increase the vitamin values. The failure of *l*-2-ketogulonic acid to cause increased ascorbic acid

TABLE III

Changes in Vitamin C Content of Tissue Slices

All aerobic experiments were done in phosphate-Ringer's solutions of pH 7.0, in an atmosphere of air, and continued for 1.0 to 1.5 hours. Most of the anaerobic experiments were done in phosphate-Ringer's solution of pH 7.0, but some were done in bicarbonate-Ringer's solution of pH 7.4. All were continued for 3 hours. Approximately 200 mg. of tissue were used per vessel. $T = 37°$. ± values represent standard errors.

Source	Tissue	Conditions	No substrate added		0.01 M glucose as substrate		Intermediates* as substrate	
			No. of experiments	Change of vitamin per gm. tissue	No. of experiments	Change of vitamin per gm. tissue	No. of experiments	Change of vitamin per gm. tissue
				γ		γ		γ
Normal rats.	Liver	Anaerobic	5	−4 ± 10	5	−11 ± 18	7	−1 ± 8
Treated " .	"	"	18	+35 ± 8	8	+11 ± 12	32	+56 ± 7
Normal " .	Brain	"	7	+9 ± 20	7	+13 ± 8	4	+10 ± 28
Treated " .	"	"	10	+13 ± 20	10	+24 ± 18	13	+32 ± 22
Normal " .	Kidney	"	5	+26 ± 7	4	+9 ± 6	10	+22 ± 4
Treated " .	"	"	9	+34 ± 17	3	+12 ± 7	35	+71 ± 7
Normal " .	Liver	Aerobic	4	+9 ± 6	5	+7 ± 14	4	−11 ± 20
Treated " .	"	"	8	+15 ± 20	6	+28 ± 7	5	−20 ± 19
Normal " .	Brain	"	4	+24 ± 24	5	−14 ± 17	4	+26 ± 11
Treated " .	"	"	6	+98 ± 16	15	+43 ± 14	5	+6 ± 11
Normal " .	Kidney	"	4	−34 ± 28	4	+5 ± 21	4	−23 ± 9
Treated " .	"	"	4	−29 ± 16	5	−40 ± 21	8	+14 ± 21
Guinea pigs..	Liver	Anaerobic	2	−4 ± 4	3	0 ± 8	4	+2 ± 7
" " ..	Brain	"	5	−3 ± 10	5	−3 ± 7	4	+13 ± 5
" " ..	Kidney	"	2	+3 ± 3	2	+5 ± 4	3	+10 ± 5
Human†.....	Liver	"	4	+23 ± 21	3	+8 ± 5	6	+31 ± 7
"	"	Aerobic					3	−37 ± 12

* Same as in Table II.

† Subject to the error caused by oxyhemoglobin. See the text.

formation was of special interest because of its close structural relation to ascorbic acid—only one step removed, and independent of further oxidation-reduction reactions. The result tends to

eliminate this product from consideration as a normal precursor of ascorbic acid in rat tissues.

An inspection of Table III reveals that, whereas normal tissues show little evidence of ascorbic acid synthesis, the evidence obtained with tissues from treated rats is considerably stronger. The variation within some of the groups was so great, as indicated by the standard error in each case, that the differences from normal

TABLE IV

Change in Ascorbic Acid Content of Muscle and Kidney Extracts and Homogenized Muscle

The tissues were removed from treated rats and ground or homogenized with twice their weight of Ringer-phosphate solution. The ground tissues were centrifuged. An amount of extract or suspension equivalent to 1 gm. of tissue was used per vessel. The pH was adjusted to 7.0 by phosphate buffer. The vessel was filled with N_2. $T = 37°$.

Tissue*	Change in ascorbic acid per gm. tissue extracted	Time
	γ	*hrs.*
Brain extract	+24	3
	+40	5
Liver "	+66	3
	+68	5
Kidney "	+30	1
	+52	3
	+95	5
Muscle "	+1	1
	+4	2
	+8	4
Homogenized muscle	+8	3
	+10	5

* In the presence of "intermediates" as substrate, defined in Table II.

are not significant, but in a few cases the difference is highly significant. It may be observed that guinea pig tissues do not show an appreciable increase, and human liver occupies an intermediate position. The human livers represent four samples, two adult and two embryonic;[3] the former contained initially 0.18 mg. of vitamin per gm. of liver and the latter 0.33 mg. These tissues differed

[3] We are indebted to Professor D. Hooker for the embryonic liver and to Dr. A. J. Bruecken for the adult liver.

from the others in that they contained large amounts of blood, and hence may have given lowered initial values (31), with a resultant indication of slight synthesis. It is recognized that the addition of metaphosphoric acid to fresh whole blood or oxyhemoglobin may result in rapid oxidation of a part of the ascorbic acid present, whereas an incubation period previous to the addition of acid could permit reduction of the oxyhemoglobin through other channels, and thus lead to an apparent increased ascorbic acid content. This source of error should not have influenced the comparative results obtained with rat tissue, however, since both normal and treated animals (and guinea pigs) were comparable. Any error that might be caused by oxyhemoglobin would be particularly low in brain tissue. The embryonic livers contained a large amount of reducing material that reacted more slowly than the vitamin. Most of the slow reduction was abolished by iodoacetamide.

Since it is difficult to prepare slices from muscle, tests were made with muscle extracts and homogenized muscle. The results from individual experiments are shown in Table IV. Comparable results with extracts of brain, liver, and kidney are also included.

DISCUSSION

If the increases in vitamin concentration reported in Tables III and IV be accepted as a measure of the actual synthesis by these tissues, one can readily calculate how much excretion of the vitamin by an intact rat could be attributed to the tissues studied. Since the excretion figures are for a 24 hour period and the longest experiments in Table III are for 3 hours, the gains in Table III would be multiplied by 8. In the best case, there would be produced about 0.45 mg. of vitamin per gm. of liver per day. A 6 gm. liver would thus make available for excretion about 2.7 mg. of vitamin. Kidneys and brain would account for about 2.0 mg. Muscle, on the basis of Table IV, might account for another 4 to 5 mg. The total for all of the body might easily reach 10.0 mg. on the basis of the *in vitro* tests, which is in good agreement with the data for ascorbic acid excretion by chloretone-fed rats (2).

A point of special interest pertains to the ascorbic acid content of the tissues from normal and treated rats, since there was clear indication of an accumulation of the vitamin in the tissues of

treated animals. The values, expressed in micrograms per gm., were as follows: from normal animals, brain (fourteen animals) 405 ± 19, liver (seventeen animals) 191 ± 22, kidney (thirteen animals) 114 ± 10; from treated animals, brain (thirty animals) 458 ± 14, liver (forty-three animals) 461 ± 19, kidney (forty-three animals) 285 ± 13. The values for normal animals are in good agreement with earlier data based upon macrotitration (32).

During the course of the investigation it was found that rat liver slices evolved appreciable quantities of hydrogen sulfide when cysteine was added as a substrate (33). Fromageot, Wookey, and Chaix (34) had made a similar observation concerning dog liver. Since the production of hydrogen sulfide might cause an appreciable error in the ascorbic acid determinations, especially in those cases in which substrates containing carbonyl groups were present, it is necessary to point out that in the present study the results obtained with brain and kidney (indicating ascorbic acid synthesis) were similar to those found with liver, although these tissues (brain and kidney) do not show appreciable production of hydrogen sulfide under the experimental conditions reported here. Furthermore, chloretone feeding did not cause an increased production of hydrogen sulfide in any of the tissues examined. Neither did the increased titration values appear to be caused by an increased concentration of sulfhydryl compounds; the velocity of the reduction and the effect of iodoacetamide were typical of ascorbic acid.

SUMMARY

A study has been made of the synthesis of ascorbic acid by slices of liver, kidney, and brain from normal rats and from rats that had been stimulated to an excretion of large amounts of ascorbic acid. A few experiments were also conducted with guinea pig tissues, human liver, extracts of rat kidney and muscle, and homogenized rat muscle. The tissues from treated rats (fed chloretone) showed evidence of synthesis *in vitro*, while those from normal rats showed distinctly less or no evidence of synthesis. Liver and kidney tissue showed greater activity than brain or muscle. Of the many substrates investigated, a mixture of pyruvate, glyceric aldehyde, and hexose diphosphate resulted in maximum synthesis. Mannose, glucose, and *l*-ketogulonic acid

were ineffective. The quantities of ascorbic acid synthesized by tissues *in vitro* were sufficient to account for a large part of the acid excreted by chloretone-fed animals.

The oxygen consumption of brain slices from chloretone-fed rats was below that found for normal animals, although the liver and kidney slices showed little or no change. The total acid production of liver slices from chloretone-treated rats was markedly low, but the brain and kidney slices showed no significant change in this respect.

The method that has been used most widely for dehydroascorbic acid estimation, based upon reduction by hydrogen sulfide to form ascorbic acid, was found to include a possible source of large errors. Many carbonyl compounds, including intermediates of normal carbohydrate metabolism and a number of quinones, combine with hydrogen sulfide to form substances that react like ascorbic acid under the conditions normally employed for titration and photoelectric colorimeter determinations. Ascorbic acid analyses that involve an initial stage of oxidation, as when mercuric salts are used for removal of sulfur compounds, would involve the same type of error.

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STUDIES ON THE MECHANISM OF HYDROGEN TRANSPORT IN ANIMAL TISSUES

V. DILUTION EFFECTS IN THE SUCCINOXIDASE SYSTEM*

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When the homogenized tissue technique was first applied to the study of animal tissue respiration, it was found that dilution of the homogenized tissue led to a marked decrease in oxygen uptake, whether endogenous, or on glucose or lactate substrates. This decrease in activity was referred to as the dilution effect (1), which was defined as the lowering of the Q_{O_2} which occurs when a homogenate is diluted. Since the Q_{O_2} is the rate of oxygen uptake per unit of tissue, the dilution effect is the disproportionality between the dilution of the enzyme preparation and the observed rate of reaction, and is considered to be an indication that the reaction being studied is catalyzed by a system which includes one or more dissociable complexes.

In contrast to the other systems studied it was found that the succinoxidase system exhibited no dilution effect. This observation was soon confirmed (2). Although the succinoxidase system was believed to include three biocatalysts (succinic dehydrogenase, cytochrome *c*, and cytochrome oxidase), it might be inferred from the data cited that all three components are combined into one undissociable complex.

Subsequent knowledge regarding the ease of extraction of cytochrome *c* from animal tissues made it appear likely that this component must readily dissociate from the succinoxidase system, and when this possibility was tested it was found (3) that the system is much less active in homogenates than in the intact

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tissue owing to a simple dilution effect which can be eliminated by the addition of cytochrome *c*. Thus the decreased activity in homogenates is not due to cell destruction *per se* but is to be correlated more properly with the dissociation constants of cytochrome *c* and its specific reaction partners. These experiments were carried out at 24°. When the temperature was increased to 38°, the Q_{O_2} of the homogenates was below the intact tissue level even in the presence of added cytochrome *c*. At about this time Axelrod *et al.* (4) found that calcium ions stimulated the succinoxidase system. Experiments showed that the calcium effect was absent or negligible at 24° but readily confirmed at 38°. Furthermore the sodium succinate which we had been using (Eastman, pure) contained traces of calcium ions. It was thus possible to understand why full activity was attained by the addition of cytochrome *c* alone at 24° but not at 38°. Apparently calcium ions are a part of the succinoxidase system but are not sufficiently dissociated to lower the rate at 24°. At 38° calcium ions and cytochrome *c* are both limiting factors in the succinoxidase system, resulting in what might be called a multiple dilution effect. However, it was found that when both of these factors are added to the system the Q_{O_2} is still below that of intact cells and a marked dilution effect remains, indicating the presence of at least one more dissociable cofactor. The demonstration of this new dilution effect and its correction are the subject of the present report.

EXPERIMENTAL

Enzyme System—Fresh rat liver homogenates were fortified with cytochrome *c* and tested as previously described. The advantages of this preparation have been pointed out (3). All experiments with the Warburg apparatus were done at 38°.

Cyanide-Succinate—This solution was prepared freshly by mixing 1.0 ml. of neutral 0.1 M NaCN with 3.3 ml. of 0.5 M sodium succinate and 2.3 ml. of water.

Sodium Succinate—Eastman sodium succinate (pure) appears to be mainly the crystalline salt ($6H_2O$) with a small admixture of the anhydrous material and some free succinic acid plus a certain amount of various elements which precipitate as the hydroxides. The commercial product was further purified by dissolving

in the minimum of hot water, adjusting to pH 8.5 with NaOH, filtering off the dark brown precipitate, and recrystallizing by adding alcohol and cooling. The crystals were sucked dry on a Buchner funnel, redissolved in hot water, recrystallized as before, and dried in air. Loss of weight at 105° was equivalent to 6.01 moles of water.

Cation Solutions—All cations were added as the chlorides prepared from reagent quality chemicals without further purification. The calcium salt was $CaCl_2 \cdot 2H_2O$.

Boiled Tissue Extract—Boiled tissue extract or *Kochsaft* was prepared by homogenizing fresh rat liver in 2 volumes of water and heating in a boiling water bath for 10 minutes with continuous stirring. The coagulum was then centrifuged without cooling, and the supernatant was filtered. The *Kochsaft* was then refrigerated until it was used, which was never more than 4 hours after preparation.

Results

Dilution Effect—The activation of the succinoxidase system by cytochrome *c* and by calcium ions gave such large increases in the rate of oxygen uptake that it became possible to test for a dilution effect in the succinoxidase system with much smaller amounts of tissue than it was previously feasible to use. Thus in the presence of an excess of calcium ions, cytochrome *c*, and sodium succinate a liver homogenate diluted so that the final solution contained only 5 mg. of fresh liver per 3 ml. gave an observed oxygen uptake of 11.3 ml. per 10 minutes. Thus a brisk oxygen uptake was noted with only 1.7 mg. of fresh liver per ml. This amount is less than one-tenth as much as the lowest level that could be studied in the absence of added cytochrome and calcium in the earlier work (1). The dilution effect in the presence of calcium as well as in its absence is clearly demonstrated in Columns 2 and 3 in Table I. Data on the Q_{O_2} in the absence of added cytochrome have been given elsewhere (3).

Effect of Aluminum—Horecker, Stotz, and Hogness (5) had reported that aluminum ions activated the succinoxidase system but this effect was not observed by Axelrod *et al.* in the system which was activated by calcium (4). Since we found a considerable dilution effect in the calcium-activated system, we tested

aluminum for its ability to correct the dilution effect and found that there was a marked increase in rate as well as a disappearance of the dilution effect, as shown by comparing Columns 3 and 5 of Table I. On the other hand aluminum ions gave no increase in activity in the absence of added calcium until the amount of tissue was reduced to the 5 mg. level (Columns 2 and 4, Table I). There was no dilution effect when only aluminum was added, although the rate was much slower than when calcium was also present. The observation by Axelrod *et al.* that aluminum did not increase the rate is thus explained, for in the absence of calcium and at the higher tissue levels their results are confirmed. The activating action of aluminum in the experiments of Horecker *et al.* can best be explained by assuming that calcium was present in their reaction mixture in amounts sufficient to make the aluminum effect possible.

Table I

Dilution Effect and Its Correction in the Succinoxidase System

The basic reaction mixture consisted of 0.8 ml. of 0.1 M sodium phosphate, pH 7.4, 0.4 ml. of 10^{-4} M cytochrome *c*, 0.3 ml. of 0.5 M sodium succinate, and H_2O to bring the final volume to 3.0 ml. after liver and ion additions. Calcium and aluminum were added as 1.2×10^{-6} mole of the chloride.

Homogenized fresh livor per flask (1)	Q_{O_2}, microliters O_2 uptako per mg. per hr. (dry weight)			
	No ion (2)	Ca^{++} (3)	Al^{+++} (4)	$Ca^{++} + Al^{+++}$ (5)
mg.				
5	18.8	45.3	34.5	83.0
10	35.4	64.2	32.2	85.8
20	34.1	71.7	28.7	81.0

Horecker *et al.* reported that chromium as well as some of the trivalent rare earths also activated the enzyme system and we have found that chromium, like aluminum, requires the presence of calcium ions in order to stimulate the succinoxidase system. Chromium also abolishes the dilution effect and appears to be completely interchangeable with aluminum. This result is shown in Table II which also demonstrates that chromium and aluminum together show no greater activation than can be obtained by

either ion alone. Yet the effect is apparently not wholly unspecific, since Horecker *et al.* reported negative results with trivalent iron. A systematic examination of the effect according to the periodic arrangement of the elements would be of interest but we have not attempted such a study.

TABLE II

Activation of Succinoxidase System by Chromium

The basic reaction mixture and ion additions were as in Table I.

Liver	Additions			Q_{O_2}
	Ca^{++}	Al^{+++}	Cr^{+++}	
mg.				
10	−	−	−	35.4
10	+	−	−	63.4
10	−	+	−	31.1
10	−	−	+	30.0
10	+	+	−	87.8
10	+	−	+	86.1
10	+	+	+	89.2
5	+	−	−	40.6
5	+	−	+	91.2
10	+	−	−	67.6
10	+	−	+	88.8
20	+	−	−	73.7
20	+	−	+	83.1*

* This slight decrease in Q_{O_2} with 20 mg. of tissue in this table and in Table III is due to the fact that the limiting rate of oxygen diffusion into the system is being approached at this concentration of tissue, as has been proved by separate experiments in which a final volume of 1 ml. was employed.

Kochsaft Experiments—The correction of the dilution effect by aluminum is perhaps stronger evidence that aluminum (or chromium) has biochemical significance than is the mere demonstration of an increased rate in the succinoxidase system. The Q_{O_2} values attained in the presence of both Ca^{++} and Al^{+++} have reached levels as high as 105 and are thus well within the range of the succinate Q_{O_2} values reported by Rosenthal (6) for liver slices at this temperature. The question which at once presents itself is whether the dilution effect which is corrected by aluminum

in reality is produced by the dissociation of aluminum ions away from active centers which they occupy in the intact cells. It was felt that a partial answer to this question might be provided by determining whether a liver *Kochsaft* could substitute for alumi-

Table III

Activation of Succinoxidase System by Liver Kochsaft

The basic reaction mixture and ion additions were as in Table I. *Kochsaft* addition, 0.8 ml. per flask.

Liver	Additions			Q_{O_2}
	Ca^{++}	Al^{+++}	*Kochsaft*	
mg.				
10	−	−	−	34.4
10	+	−	−	70.2
10	−	+	−	36.6
10	+	+	−	86.8
10	−	−	+	86.9
10	+	−	+	92.2
10	−	+	+	91.3
10	+	+	+	99.8
0	−	−	+	2.8*
10	−	−	+	7.9†
0	+	+	+	1.0*
10	+	+	+	7.3†
5	+	−	−	25.8
5	−	−	+	94.2
10	+	−	−	61.1
10	−	−	+	90.8
20	+	−	−	77.5
20	−	−	+	83.3‡

* Q_{O_2} calculated as for 10 mg. of liver.

† No succinate added.

‡ See foot-note to Table II.

num in the succinoxidase system. Direct chemical analysis for traces of aluminum was not attempted, but Horecker *et al.* reported spectrometric evidence for its presence in the system which they studied. *Kochsaft* was studied both from the standpoint of its activating action and its ability to eliminate the dilution effect. The results are shown in Table III. It is apparent that the *Kochsaft* can substitute for both calcium and aluminum ions.

Moreover it seems reasonable to conclude that the activating action of the *Kochsaft* can be explained solely on the basis of the calcium and aluminum ions or similar ions which it contains. With one exception the literature on succinic dehydrogenase is an unbroken series of negative attempts to activate the succinoxidase system with *Kochsaft*. Ahlgren (7), who has reviewed this literature, reported activation with a *Kochsaft* but his results were minimized by Hopkins *et al.* (8), who obtained negative results with *Kochsaft* in confirmation of the earlier literature.

Spontaneous Activation—Elliott and Greig (2) observed that there was frequently a spontaneous increase in succinoxidase activity when a homogenate was allowed to stand in the cold for several hours, and they found it necessary to determine the activity of the preparation both when it was fresh and after standing. They attributed the increase to the further dispersion of the components of the succinoxidase system. This explanation did not seem entirely reasonable, since further solution of any component should lead to a dilution effect. From the fact that the calcium effect was difficult to demonstrate at 24° but readily apparent at 38° we concluded that the dissociation constant of the hypothetical calcium complex in the succinoxidase system must be exceedingly small. Therefore the spontaneous activation might be due to a dissociation of calcium from its various combinations together with a migration to the complexes having smaller dissociation constants. The succinoxidase system was presumed to fall in the latter category. This idea was tested by comparing the spontaneous activation in the presence of calcium as well as in its absence. The results are shown in Table IV. The spontaneous activation observed by Elliott and Greig was confirmed but it was found that when the system was studied in the presence of added calcium no increase in activity was effected by standing and the calcium-activated system surpassed the spontaneously activated system. Aluminum may enter into the effect in an analogous manner, although it appears that the spontaneous activation is due mainly to the liberation of calcium from other tissue complexes. It is apparent that the succinoxidase system can be brought to maximal activity by the addition of calcium and aluminum ions, and the spontaneous activation becomes unnecessary.

Locus of Ion Effects—Horecker *et al.* (5) were unable to show the aluminum effect on any of the components of the succinoxidase system, and they suggested that it was in some way connected with the formation of a precipitate which could be seen when larger amounts of aluminum were added to the reaction mixture. However, this explanation would seem to be invalidated by the fact that the aluminum effect is dependent upon the presence of traces of calcium ions.

TABLE IV

Rôle of Calcium in Spontaneous Activation of Succinoxidase System

The basic reaction mixture and ion additions were as in Table I.

Homogenate No.	Q_{O_2} (fresh)		Q_{O_2} (after 4-6 hrs.)	
	Without Ca^{++}	With Ca^{++}	Without Ca^{++}	With Ca^{++}
724A	28.3	48.0	40.7	48.3
724B	14.9	53.7	33.3	46.5
724C	26.1	57.0	38.5	55.8
725A	24.6	59.9	38.5	57.9
725B	27.5	62.9	38.7	55.0
725C	28.4	52.7	43.3	52.9
728A	22.2	55.6	47.5	60.8
728B	31.0	67.9	49.5	63.7
728C	20.8	78.2	45.6	61.0
84A	49.1	95.0	57.6	87.0
84B	29.3	45.4	34.2	31.9
84C	33.2	63.6	42.2	51.1
Average.....	28.0	61.7	42.5	56.0

In their attempt to localize the action of aluminum, Horecker *et al.* had studied the dehydrogenase by the methylene blue technique. It is interesting to note that Ahlgren as well as other workers likewise obtained negative results when they attempted to demonstrate the action of *Kochsaft* on this system by the methylene blue technique. On the basis of these findings and our own experience with methylene blue (3) we studied the dehydrogenase on the basis of cytochrome reduction rather than dye reduction.

10 per cent liver homogenate was centrifuged at 3000 R.P.M. for 10 minutes and the filtered supernatant, although it contains

less than 5 per cent of the original activity, was used as a source of enzyme, because it consists of only the smallest particles of the dehydrogenase (3). The reaction mixtures were made up in test-tubes and the reaction was initiated by adding 0.2 ml. of cyanide-succinate. After mixing, the solution was transferred to the absorption cell and the rate of reduction of cytochrome *c* was measured in the photoelectric spectrophotometer (9). The cyanide was used to prevent reoxidation of the reduced cytochrome *c*. It was found that the rate could sometimes be increased by the addition of both calcium and aluminum ions but since the

TABLE V

Effect of Ca and Al Ions on Cytochrome c Reduction

The rate was measured at room temperature. The reaction mixture consisted of 1.0 ml. of 0.1 M sodium phosphate, pH 7.4, 0.5 ml. of 10^{-4} M cytochrome *c*, 1.25 ml. of H_2O (part of which was displaced by 0.3 ml. of 4×10^{-3} M Ca^{++} and 0.3 ml. of 4×10^{-3} M Al^{+++} solutions, as indicated), and 0.05 ml. of the enzyme preparation; 0.2 ml. of cyanide-succinate was added to initiate the reaction. Rates were measured in the order indicated.

Test No.	Basic system	Addition	Rate of cytochrome *c* reduction, ΔE_{550} per min.
1	Fresh reaction mixture		0.068
2	Reaction mixture as in (1) incubated 30 min. at 38°, cooled		0.042
3	Same	$Ca^{++} + Al^{+++}$	0.082
4	"	" + "	0.072
5	"		0.036

reaction was carried out at room temperature the small dissociation of the ions made the demonstration difficult. However, by heating the reaction mixture to 38° for a short time and then cooling to room temperature with the enzyme at high dilution a loss of activity resulted which could be restored by the addition of calcium and aluminum. The inactivation could also be effected by storing the reaction mixture containing the enzyme at high dilution for several hours at 0°. The lability of the enzyme makes these experiments rather troublesome. Aluminum ions alone appear to be more effective than calcium ions alone. The results of a typical experiment are shown in Table V.

More striking evidence than is given in Table V can be observed visually simply by noting the color change in the various flasks in a Warburg experiment such as is described in Table I, with 10 mg. of tissue per flask. If the Warburg flasks are shaken so as to oxidize all of the cytochrome and then allowed to remain stationary, the rate of diffusion of oxygen into the medium becomes limiting in the flasks which contain calcium and aluminum, but not in the flasks without these ions; the former turn pink, showing that the cytochrome is reduced, while the latter retain the color of oxidized cytochrome.

Since the earlier investigators (5, 7) obtained positive results with the over-all system in which cytochrome *c* was an intermediary but negative results when methylene blue was the end-acceptor and since we have obtained positive results with cytochrome *c* as the end-acceptor, it seems reasonable to suggest that the locus of the aluminum effect may be the reaction between cytochrome *c* and its reductant, which is tentatively considered to be succinic dehydrogenase (see the discussion).

Work in this laboratory[1] has shown that fluoride inhibits succinic dehydrogenase competitively with respect to succinate, and thus the locus of the fluoride action is apparently the reaction between succinate and its dehydrogenase. The insolubility of calcium fluoride suggests that fluoride may act by binding calcium. From this it would follow that the locus of the calcium effect may be the reaction between succinate and the dehydrogenase.

DISCUSSION

The fact that we have been able to demonstrate the effect of calcium and aluminum in the partial reaction involving cytochrome *c* reduction, while others have been unable to demonstrate activation by cofactors in the partial reaction involving dye reduction, again emphasizes the point which we have previously stressed (3); namely, the desirability of using the physiological carrier in studies of this type.

The data illustrate the importance of the dilution effect as a tool for the study of the mechanism of hydrogen transport. In

[1] D. J. O'Kane, unpublished data.

the present case the existence of a dilution effect was taken as evidence that at least one more dissociable factor is needed in addition to cytochrome *c* in order to complete the succinoxidase system, and previous data (4, 5) made possible the identification of both calcium and aluminum ions as components of the system. These results led to the demonstration of a *Kochsaft* effect in the succinoxidase system. Our data do not prove that calcium and aluminum are the factors present in the *Kochsaft* but the evidence is compatible with this view. Horecker (10) has shown that under certain circumstances malonate and some other compounds will stimulate the succinoxidase system if they are added in small concentrations. This effect was attributed to the toxic action of traces of copper which were introduced during the preparation of the enzyme. Our enzyme preparation is carried out in an all-glass apparatus (1) and apparently does not contain sufficient copper to affect the rate. The preparation is not stimulated by malonate at any concentration, and toxicity due to malonate could be demonstrated at concentrations as low as 10^{-4} M. Although copper toxicity might readily explain the dilution effect and its correction by a *Kochsaft,* we have not been able to obtain any evidence in support of the latter view.

From the data presented it might be suggested that there are no more dissociable factors in the succinoxidase system as employed here. This is rather surprising in view of the fact that magnesium ions have been shown to stimulate the system (11). Furthermore the fact that phosphate esterification has been coupled with succinate oxidation introduces the possibility that additional factors are a part of the system. Nevertheless the data presented prove that if these factors are a part of the system they are relatively undissociated in the diluted liver homogenate.

According to the data available thus far we may describe the succinoxidase system as consisting of succinate, calcium ions, succinic dehydrogenase, aluminum ions, cytochrome *c*, cytochrome oxidase, and oxygen. In this system, the dehydrogenase and the oxidase are in the form of particulate matter (3). The data presented do not prove that the biocatalysts listed are the only components of the system, nor that succinic dehydrogenase reacts directly with cytochrome *c*. It is apparent, however, that if an additional biocatalyst is interposed at this point it is rela-

tively undissociated from the dehydrogenase at the dilutions employed. Similarly if cytochrome oxidase consists of more than one entity, the respective biocatalysts must be relatively undissociated from the oxidase. The loss in succinoxidase activity which results when intact liver cells are homogenized is apparently a simple dilution effect due to the dissociation of one component (cytochrome *c*) from the system at 24° (3), but is apparently a multiple dilution effect at 38°, at which temperature three components dissociate from the system; namely, cytochrome *c*, calcium ions, and aluminum ions or their equivalents. The particles which we identify with the dehydrogenase and the oxidase are probably identical with those which have been mentioned by various workers including Stern (12) who states, "we believe that the active groups of the various component catalysts are arranged in or on them [the particles] in an orderly fashion so as to ensure a smooth functioning of the highly complex process of cellular respiration." Although this concept may be correct, the available data do not even prove that succinic dehydrogenase and cytochrome oxidase are united into one structural unit, since they could be widely separated with random distribution and still show no dilution effect provided that a sufficient amount of the dissociable connecting carrier (*i.e.*, cytochrome *c*) is added to keep both the dehydrogenase and the oxidase effectively saturated with the carrier.

It seems likely that the test system described represents a complete succinoxidase system, which can be used as a measure of the succinoxidase activity of animal tissues or for the study of various toxic substances.

SUMMARY

1. A dilution effect was demonstrated in the succinoxidase system in a rat liver homogenate which had been fortified with cytochrome *c*.

2. The effect was corrected by the addition of calcium and aluminum ions together, or of a liver *Kochsaft*.

3. Chromic ions were interchangeable with aluminum ions in the system.

4. The ion effects were shown to occur in the partial reaction ending in cytochrome *c* reduction.

5. The loss in succinoxidase activity which occurs when liver is homogenized and diluted is considered to be a multiple dilution effect, caused by the dissociation of cytochrome *c*, calcium ions, and aluminum ions (or their equivalents) away from succinic dehydrogenase and cytochrome oxidase.

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THE RELATION OF THE PH OF INTESTINAL CONTENTS TO CALCIUM AND PHOSPHORUS UTILIZATION

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Several theories have been advanced to explain the manner in which vitamin D-free fats can beneficially influence calcification. Recently the writer (1) published data showing that none of these explanations, with the possible exception of the one given by Boyd, Crum, and Lyman (2), was satisfactory. Boyd *et al.* presented evidence indicating that fats make the contents of the intestines more acid, thereby increasing the absorption of calcium and phosphorus and facilitating bone formation. In an attempt to determine the validity of this suggestion the influence of a number of substances on the acidity of the intestinal contents and simultaneously on calcification has been studied. The results of these experiments are presented below. Numerous investigators have studied the relation of the pH of intestinal contents and feces to calcification, especially in regard to the development or prevention of rickets. As this subject has been reviewed at different times, no attempt to cover the literature is made here.

EXPERIMENTAL

Albino rats which were raised in the laboratory and taken from litters which had been reduced to six in number about the 2nd day of life were used as experimental animals. They were put on the various diets to be investigated at 25 days of age (occasionally 24 or 26 days). In the great majority of experiments a comparison was made between two diets, and for this purpose three animals of one litter were given one diet and the remaining three the other diet. After 21 days on the experiment (in a few cases 20 days) the rats were anesthetized with ether and the intestinal tract including

the cecum and colon was removed. The small intestines were divided into four approximately equal segments. The contents of each segment, cecum, and colon were squeezed out, and treated separately. The contents of corresponding segments, ceca, and colons from all animals of any one group were combined, 2 ml. of distilled water were added, and the contents well agitated with a stirring rod. The insoluble portion was separated by centrifugation with decantation of the supernatant liquid. It was usually necessary to add 5 ml. of water to the contents of the ceca and colons to insure separation of sufficient liquid. The pH determination on the supernatant liquid was made with a Leeds and Northrup glass electrode pH meter. A special microelectrode made possible the determination on 1 ml. of solution. The determinations of pH were done as quickly as possible after the samples were taken, and in most cases the determinations on the two groups of animals comprising any one experiment were carried out on the same day. In addition to determining the pH on the intestinal contents, the right femur of each animal was removed and the percentage ash on the lipid-free bone determined. The degree of rickets was determined by examining the distal end of at least one radius from each animal by the "line test" technique. The degree of rickets was arbitrarily expressed in numbers from 1 to 10, 1 indicating the first detectable widening of the uncalcified cartilaginous disk and 10 indicating a very wide uncalcified area with straight regular margins.

Except for the salt mixture the basal diet used was nearly constant throughout and was composed of the following ingredients expressed in per cent: alcohol-extracted fibrin 18, dried brewers' yeast 5, Salt Mixture 5 (3) 5, or Salt Mixture 10 (4) 3, and dextrinized starch to 100. 5 drops of S. M. A. carotene in oil were added to each 100 gm. of diet. In a few cases glucose (cerelose) was used in place of dextrin and also in some experiments 2 per cent of agar was used as roughage. All additions to the basal diet were made at the expense of the carbohydrate. When fatty materials such as lard, oleic acid, or sodium oleate were given, the other constituents were increased (except the carbohydrate which was correspondingly reduced) to allow for the increase in energy content.

The results of the experiments are summarized in Table I and

TABLE I

Influence of Various Dietary Supplements on pH of Intestinal Contents and Calcification of Bone

Series No.	Salt mixture No.	Dietary supplement	No. of animals	No. of determinations	pH of intestinal contents: Segments of small intestines, 1st	2nd	3rd	4th	Cecum	Colon	Rickets	Femur Ash, mg.	Femur Ash, per cent
1	5	Lard	24	8	6.65	6.57	6.56	7.61	6.98	6.63	4.3	22.7	27.1
2	5	None	24	8	6.46	6.54	6.73	8.00	6.90	6.77	8.7	17.6	22.3
3	5	Oleic acid	21	7	6.72	6.77	6.76	7.76	6.85	6.78	2.7	26.6	31.2
4	5	None	21	7	6.61	6.75	7.10	8.31	7.14	6.97	8.5	19.3	23.7
5	5	Irradiated ergosterol*	12	4	6.87	6.82	6.81	7.97	6.69	6.42	0	35.9	34.2
6	5	None	12	4	6.73	6.56	6.79	8.24	7.00	6.81	8.0	19.2	23.2
7	10	1.7% K_2HPO_4	3	1	6.63	6.78	7.03	8.37	7.00	7.01	0	58.9	42.3
8	10	None	3	1	6.67	6.60	7.09	8.37	7.14	6.90	4.7	20.2	23.2
9	5	2.25% K_2HPO_4	3	1	6.43	6.70	7.12	8.28	6.82	7.15	0	59.3	44.4
10	5	None	3	1	6.70	6.65	6.90	8.28	6.98	7.20	7.3	15.9	20.3
11		2.5% Al sulfate†	3	1	6.94	6.70	6.91	8.31	7.13	6.34	7.0	16.2	21.2
12		5% Al sulfate†	6	2	7.13	7.17	7.18	8.08	7.23	6.78	7.0	12.5	18.8
13		Stock diet	8	3	6.80	6.74	6.50	7.67	6.85	6.65	0	79.0	47.9
14	10	2% NH_4Cl	6	2	6.49	6.53	6.75	8.36	6.63	6.60	8.8	20.5	21.2
15	10	None	6	2	6.62	6.61	6.82	7.99	6.81	6.74	8.2	19.2	19.0
16	10	4% NH_4Cl	6	2	6.40	6.49	7.15	8.22	6.67	6.59	8.3	21.3	25.9
17	10	None	6	2	6.68	6.63	7.05	8.04	7.02	6.99	7.8	18.5	22.8
18	13‡	$CaCl_2$	9	3	6.43	6.53	7.10	8.49	6.96	7.09	9.0	19.2	22.8
19	10	None	9	3	6.59	6.48	6.95	8.26	6.88	6.81	9.0	21.1	24.0
20	10	5% triacetin	9	3	6.60	6.51	7.10	8.26	6.87	6.92	8.0	20.5	24.3
21	10	None	9	3	6.57	6.55	6.76	7.91	6.72	6.66	6.6	20.4	23.7
22	10	10% triacetin	3	1	6.23	6.59	6.40	7.88	6.77	6.60	7.0	19.5	22.8
23	10	None	3	1	6.46	6.48	6.46	8.04	6.92	6.75	9.7	19.3	22.4
24	10	20% triacetin	3	1	6.53	6.77	6.97	8.36	6.79	6.90	9.3	19.1	22.0
25	10	None	3	1	6.66	6.74	6.74	8.20	6.91	6.61	8.7	17.5	20.4
26	5	Lactose	15	5	6.42	6.53	6.91	7.74	6.23	6.32	7.7	24.1	28.0
27	5	Lard	15	5	6.58	6.50	6.48	7.84	6.58	6.15	2.8	29.0	29.3
28	5	Oleic acid	9	3	6.69	6.76	6.74	7.86	6.64	6.50	4.7	24.6	26.5
29	5	Na oleate	9	3	6.57	6.62	6.69	8.16	6.84	6.86	8.1	19.3	22.7

* 100 I.U. of vitamin D per 100 gm. of diet.

† $Al_2(SO_4)_3 \cdot 18H_2O$ given in addition to stock diet.

‡ Calcium carbonate of Salt Mixture 10 was replaced by an equivalent amount of calcium chloride.

presented as averages. That is, for example, all animals which received lard and were compared directly with litter mates on the basal diet are designated as Series 1, while the animals which served as controls for Series 1 are Series 2.

For the most part the basal diet given above, with either Salt Mixture 5 or 10, served as the control with which to compare the effect of an added substance. In one group of experiments, however, the effect of lactose was compared with lard and in another oleic acid was compared with sodium oleate. In the experiments in which aluminum sulfate was added the dry portion of the Steenbock stock diet (5) modified to contain 2 per cent of yeast was used in place of the synthetic diet.

In Series 1 and 2 (Table I) 10 per cent of lard was compared with the basal diet. The lard produced a slight but definite increase in calcification, as shown by the absolute and relative amounts of femur ash and also by the degree of rickets. There was no consistent difference in pH except in the fourth segment of the small intestines. Not only do the averages show a difference but in only one of the eight trials were the contents of the fourth segment from the animals on the basal diet more acid than the contents of the corresponding segment from the litter mates receiving the lard. In this one case the contents from the animals on the basal diet were exceptionally acid.

In Series 3 and 4 the effect of 10 per cent oleic acid was studied with results almost identical with those obtained with lard. In this series of seven trials there was no case in which the animals on the basal diet showed as low a pH of the contents of the fourth segment of the small intestines as did the animals receiving the oleic acid.

The effect of vitamin D (irradiated ergosterol[1]) is shown in Series 5 and 6. In this case calcification was very much increased and here again the only consistent difference in pH was in the fourth segment. As with oleic acid, there was no case in which the control animals had as low a pH in this segment as the litter mates receiving vitamin D. The averages for the contents of the cecum and colon are slightly more acid for the irradiated ergosterol group than for the controls. The same was true for the animals

[1] Furnished through the courtesy of Dr. Warren M. Cox, Jr., Mead Johnson and Company, Evansville, Indiana.

fed oleic acid but in neither case was this difference entirely consistent in respect to the individual experiments comprising the series.

Series 7 to 10 included only a small number of animals but nevertheless they show rather clearly that added phosphorus had no definite effect on the acidity of the intestinal contents, although it greatly increased calcification. This is in agreement with Shohl and Bing (6) who found that alkaline phosphate although producing healing of rachitic lesions did not make the feces more acid.

It has been repeatedly shown that a good calcifying diet can be made rachitogenic by the addition of a salt of a metal which forms an insoluble phosphate. Series 11, 12, and 13 show the result of adding 2.5 and 5 per cent aluminum sulfate to the stock diet referred to above. The addition of this salt in either amount considerably reduced calcification and produced a greater alkalinity of the contents of the fourth segments. It also appears that the larger amount of the aluminum salt slightly increased the alkalinity throughout the greater part of the intestinal tract. This is somewhat surprising in view of the acid nature of aluminum sulfate, but is in conformity with the findings of Karr and Abbott (7). These authors observed that various substances, including acids, upon entering the small intestines stimulate the secretion of sodium bicarbonate, which prevents a decrease in pH and may even make the contents more alkaline.

If this is really the case, a salt such as ammonium chloride which gives an acid reaction but does not form an insoluble phosphate should neither make the intestines more acid nor influence calcification. In the experiments presented as Series 14 to 17 this seems to be the case. In these experiments Salt Mixture 10 (instead of Salt Mixture 5) with its lower content of calcium carbonate was used to reduce the effect of the salt mixture in neutralizing the acidity of the ammonium chloride. These findings are in agreement with those of Oser (8) who has shown that substances such as sodium carbonate and ammonium chloride do not change the pH of the intestinal contents or influence the development of rickets. The replacing of the calcium carbonate (Series 18 and 19) with an equivalent amount of calcium chloride likewise had very little influence on either the pH of the intestines or on calcification. Ammonium chloride and calcium chloride

appear to have made the intestinal contents slightly more acid in the upper part and very slightly more alkaline in the lower part of the small intestines. It was somewhat unexpected to find that both lard and oleic acid decreased the alkalinity of the lower part of the small intestines and also increased calcification but a salt such as ammonium chloride did neither. It was thought that possibly this difference was due not only to the effect of ammonium chloride on bicarbonate secretion but also to the rapid absorption of this simple water-soluble salt in the upper part of the intestines. To test this idea further, triacetin was given in varying amounts. This substance would give rise to a water-soluble acid but only after hydrolysis, which would probably produce an acidity in a more distal portion of the small intestines. The triacetin was given at levels of 5, 10, and 20 per cent and compared with the basal diet containing Salt Mixture 10. The latter was used as before to reduce the neutralizing effect of the inorganic mixture. The data presented in Series 20 to 25 show clearly that this substance had no demonstrable effect on either the pH of the intestinal tract or calcification. Deuel, Hallman, and Reifman (9) have recently shown that fatty acids with an uneven number of carbon atoms are more slowly absorbed than those with an even number of carbon atoms. In the belief that the acetic acid of triacetin still might have been too quickly absorbed to have any effect tripropionin was tried. So far these experiments have been unsatisfactory, as the animals ate but small amounts of the diet containing the tripropionin.

It is a well known observation that lactose facilitates utilization of calcium (10) and also increases the acidity of the intestinal tract (11). In several experiments the effect of lactose on intestinal acidity and calcification was compared with lard. This was done in an attempt to determine whether a given decrease in pH caused by either of these two dietary supplements would in each case produce approximately the same increase in calcification. The lactose was fed at levels varying from 25 to 70 per cent of the diet. Only those results obtained with the lowest level are reported, as the higher levels produced marked diarrhea and distention of the intestinal tract with decreased food consumption and poor growth. The lard was fed at only one level (10 per cent) with adjustments being made in the other constituents of the diet as previously explained to allow for the differ-

ence in the caloric content of the lactose and lard. The results of these experiments are presented in Series 26 and 27. As may be seen, there was little difference in the pH of the intestinal contents. If anything the lactose produced slightly more acidity than the lard. In spite of this the lard definitely produced more calcification than did the lactose. This is particularly noticeable in the weight of femur ash and in the degree of rickets. The close agreement in the percentage of ash, even though there was a definite difference in the absolute amounts, is in all probability due to the slower rate of growth of the animals receiving the lactose. The average gain in weight of these animals was 17.7 gm. for the 21 day period and 29.0 gm. for the animals receiving the lard. This greater gain in weight may have also accounted for the increase in weight of bone ash for the animals on the lard diet but the line test definitely indicated that lard had a greater antirachitic action than did the lactose.

Finally in two more series of experiments (Series 28 and 29) oleic acid and sodium oleate were compared. They were both fed at the 10 per cent level. It was previously reported that sodium oleate had no antirachitic action, whereas oleic acid did. This observation was confirmed, and here again the only consistent difference in pH was found in the fourth segment of the small intestines. This is surprising, as it would be impossible for much free oleic acid to exist in solution at the pH found in any region of the intestinal tract. Probably the greater part of the fatty acid was not in solution or was in combination with bile salts. This phase of the problem is being investigated further.

DISCUSSION

When these data are considered as a whole, the high pH of the distal one-fourth of the small intestines is outstanding. Of approximately 100 series of pH determinations (representing nearly 300 animals) which have been made there was not a single instance in which this segment of the small intestine did not show a higher value than any other portion of the whole intestinal tract. Oser (8) found the greatest alkalinity in the cecum but his values for the lower ileum were considerably below those reported here.

It seems improbable, however, that these high results are er-

roneous, for not only were they entirely consistent, but they were obtained in each case on the combined contents taken from more than one animal (usually three) and after the addition of only 2 ml. of water. In several experiments, after determination of the pH of the intestinal contents to which 2 ml. of water had been added, the solutions were further diluted with several ml. of water and the pH again determined. In most cases the additional water produced little or no change in pH. Furthermore, the error, if any, caused by simple dilution would have been toward the acid side. Eastman and Miller (12), using the glass electrode, determined the pH of the contents of the gastrointestinal tract of a large number of rats. They divided the small intestines into three segments and consistently found the lower third to be the most alkaline portion of the whole tract. Their values are somewhat below those presented above. This difference may be due, at least in part, to the greater length of the small intestines (one-third instead of one-fourth) which they included in the lower segment.

When analyzed from the standpoint of the relation of intestinal pH to calcification, these data are still not conclusive. If one considers the first two experiments, that is the comparison of lard and oleic acid with the basal diet, it is conceivable that there is a sufficient difference in the average pH of the lower one-fourth of the small intestines (and possibly the cecum and colon) to account for the slight increase in calcification on either the lard or oleic acid as compared to the controls. A difference in pH of only a few tenths of a unit in the region of 7.5 to 8 changes considerably the ratio between the diphosphate and monophosphate ions. To accept this explanation, it is necessary to assume that practically all the absorption of calcium or phosphorus or both takes place in the lower part of the intestinal tract, for it is only in this portion that a consistent difference in pH was observed. Furthermore, all other portions of the intestinal tract of the control animals were more acid than the lower ileum of the animals on the supplemented diets. This should have facilitated absorption in these regions of the control animals if pH is the only factor involved. It should also be pointed out that in these two groups of experiments, though the acidity of the lowest segment of the small intestines of the animals on the supplemented diets (with only one exception) was greater than that of the litter

mate controls, this difference was not so consistent when the results of one trial were compared with those of another. That is, although the pH of the lower ileum of the animals receiving the dietary supplement was lower than that of their controls run simultaneously, it was not always lower than that of other groups of controls determined at different times. On the other hand, as mentioned above, there was a tendency for an increase in acidity of the cecum and colon of the animals on the supplemented diets, especially oleic acid. Bergeim (10) has shown that phosphorus is reabsorbed from the colon and the amount of reabsorption is influenced by the pH. It is possible that the increased acidity of the colon might also have been a factor in these experiments.

Additional doubt is thrown on the acid theory when the results with vitamin D are considered. Here again, as pointed out, the lower intestines were more acid on the supplemented than on the control diets. However, in this case the change in acidity caused by vitamin D was no greater than that resulting from the lard or oleic acid, but the amount of calcification was a great deal more. This would seem to eliminate definitely a decrease in pH of the intestinal contents as being the principal method by which the antirachitic factor functions in augmenting calcification of the growing bone. Kline, Keenan, Elvehjem, and Hart (11) working with chicks also found that lactose decreased the acidity of the intestinal tract to a greater extent than did direct irradiation of the animals with ultraviolet light. The latter, however, produced considerably more calcification.

Although aluminum sulfate made the intestinal contents slightly more alkaline, its marked effect in diminishing calcification cannot be explained on this basis. The next group of experiments (those with added phosphate) shows that in the presence of ample phosphorus rickets does not develop even though the intestines have a high pH.

As neither pH nor calcification was influenced by calcium chloride, ammonium chloride, or triacetin, the experiments with these substances gave no information on the relation of the acidity of the intestinal tract to calcification. The experiments with lactose, on the other hand, indicate that a simple increase in acidity comparable with that produced by lard is insufficient to produce a definite antirachitic action.

Although not definitely eliminating an increase in acidity as

the method by which fats increase calcification, these data when viewed as a whole throw considerable doubt on this theory. The manner in which fatty substances do increase calcification is still obscure. It is interesting to recall the observation of Patwardhan and Nhavi (13) that phosphorus is absorbed by way of the lymph. The increased fat in the diet may in some manner increase the absorption of phosphorus and possibly also calcium by this route. Von Beznák (14), however, believes that the amount of calcium absorbed by way of the lymph is insignificant.

SUMMARY

The effect of a number of substances on the pH of the intestinal contents of rats and simultaneously on calcification of bone has been studied. The intestinal tract was divided into six parts—four equal segments of the small intestines, cecum, and colon. The contents from corresponding parts of several animals (usually three) were united and the pH of each determined with a glass electrode pH meter. The following observations were made.

1. The most alkaline portion of the intestinal tract was the distal one-fourth of the small intestines.

2. When given in addition to a rachitogenic diet, lard, oleic acid, and vitamin D all increased the acidity of the lower portion of the ileum and less consistently increased the acidity of the cecum and colon. These same substances also showed definite antirachitic action which was much greater on the part of vitamin D than either of the other two, although vitamin D did not produce a lower pH in the intestinal tract than did lard or oleic acid.

3. The addition of aluminum sulfate to a stock diet resulted in severe rickets and a definite increase in pH throughout the greater part of the intestinal tract.

4. The addition of dibasic phosphate to a low phosphorus rachitogenic diet protected the animals against rickets without changing the pH of the intestinal tract.

5. Ammonium chloride, calcium chloride, and triacetin were without effect on either the pH of the intestinal contents or calcification.

6. Lactose produced as much acidity in the lower intestinal tract as did lard but it did not show any antirachitic action.

7. Although there was no difference in the pH of the upper portions of the small intestines when oleic acid and sodium oleate were compared, the acid produced the greater acidity in the lower ileum and also exhibited a definite antirachitic action, whereas the soap did not.

These findings are discussed from the standpoint of the relation of the pH of the intestinal contents to bone calcification. These data do not definitely eliminate an increase in acidity as the method by which fats beneficially influence calcification but they do throw considerable doubt on this theory.

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EQUILIBRIA IN DIASTATIC REACTIONS*

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The erroneous view, that the end-product of diastatic reactions is maltose, is so firmly established that, when measuring diastatic activity by the reducing power of the reaction products, most investigators compute the reduced copper as maltose. Others follow quantitative changes by the polarimetric method, again on the premise that any change in the optical activity of reaction mixtures is due exclusively to the conversion of starch to maltose. In the light of both old and new observations, this concept is fallacious, since the products of diastatic reactions include glucose as well as an array of non-fermentable substances (dextrins) possessing copper-reducing power (1–3) and optical activity that differ from the properties of both starch and maltose.

In a previous report from this laboratory (4) it was pointed out that this lack of regard for well established facts has become the source of much confusion with respect to the kinetics of diastatic action. In the present article, data are presented to show the error in the generally adopted view that in diastatic reactions equilibrium is reached when 80 to 85 per cent of the starch is converted to maltose, and that at this point the reaction is virtually stopped by the formation of a dextrin (grenzdextrin), which is resistant to further enzymatic action. While this concept is still presented in modern text-books and monographs, one finds in the extensive literature on the subject numerous observations showing equilibria at considerably lower as well as at higher conversion levels. Fernbach and Wolff (5), for example, obtained diastatic conversions as high as 103.8 per cent, calculated as maltose, concluding from this result that the starch was completely converted to maltose.

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Technique

In the present studies of equilibria the method of tripartite analysis of diastatic reaction products (6) was employed. Starch pastes prepared from unmodified rice or corn-starch were used as substrates, and saliva and barley malt as the sources of diastase.

Diastase was precipitated from human saliva with 4 volumes of alcohol and separated by centrifugation. The supernatant fluid was discarded and the precipitate was dissolved in 0.25 per cent sodium chloride solution. A fraction that had become insoluble through contact with alcohol was eliminated by centrifugation. Precipitation with alcohol was repeated and the precipitate was dehydrated, being washed first with alcohol and then with ether. The final product, a dry powder, was kept in a desiccator. For use in experiments, the desired amount of the powder was extracted with cold 0.25 per cent sodium chloride solution; insoluble particles were removed by centrifugation. Occasionally, saliva itself was used as the enzyme solution; for this purpose the saliva was first centrifuged to remove the mucus, and the supernatant fluid was passed through a Berkefeld filter.

When barley malt was used as the source of diastase, the malt was ground to a fine flour and extracted with cold 0.25 per cent sodium chloride solution. This extract was precipitated with 4 volumes of alcohol and the precipitate was treated in the same manner as that obtained from saliva.

In all experiments the reaction mixtures contained 0.25 per cent sodium chloride; the pH was approximately 6.8; the temperature was 40°. Toluene was added as preservative, and it was ascertained that no bacterial contamination had occurred during the period of incubation. The samples, periodically withdrawn for analysis, were rapidly heated to a point near boiling and further heated for about 5 minutes in a boiling water bath, in order to stop enzyme action.

Apparent Equilibria—In Table I are presented three experiments, the results having been obtained by the traditional analytical procedure; that is to say, by computing the total copper-reducing power of the reaction products as maltose. The reaction was allowed to proceed until reducing power ceased to show any changes; *i.e.*, until the reaction had apparently reached a state

of equilibrium. As may be seen, this occurred in Experiment I when 51 to 52 per cent of the substrate appeared as "maltose,"

TABLE I

Pseudoequilibria in Diastatic Reactions

The maltose equivalents of the copper-reducing power are given per liter of reaction mixture.

Experiment No.		Duration of reaction	"Maltose" formed	Starch equivalent of "maltose"*	Starch converted
		hrs.	*mg.*	*mg.*	*per cent*
I	3.0 gm. starch, 2.5 gm. NaCl, and 0.4 gm. diastase per liter reaction mixture; 1 mg. diastase per 7.5 mg. starch	1.0	1057	1004	33.5
		2.0	1336	1269	42.3
		3.0	1490	1415	47.2
		18.0	1619	1538	51.3
		24.0	1622	1541	51.4
		48.0	1650	1567	52.2
		72.0	1634	1552	51.7
II	0.418 gm. starch, 2.5 gm. NaCl, and 0.278 gm. diastase per liter reaction mixture; 1 mg. diastase per 1.5 mg. starch	0.2	357	339	81.0
		0.4	357	339	81.0
		0.8	369	351	84.0
		1.0	375	356	85.4
		2.0	390	370	88.5
		3.0	390	370	88.5
		18.0	462	439	105.0
		24.0	471	447	107.0
		48.0	479	455	108.8
		72.0	490	465	111.2
III	0.418 gm. starch, 2.5 gm. NaCl, 0.278 gm. diastase, and 200 cc. filtered saliva per liter reaction mixture	0.2	451	428	102.0
		0.4	457	434	104.0
		0.8	512	486	116.0
		1.0	515	489	117.0
		2.0	536	509	122.0
		3.0	536	509	122.0
		18.0	531	504	120.0
		24.0	544	517	123.0
		48.0	481	457	109.0

* Starch equivalent of maltose = maltose × (324/342). $2C_6H_{10}O_5 = 324$; $C_{12}H_{22}O_{11} = 342$.

while in Experiments II and III the conversion ratios were about 111 and 120 per cent, respectively; in all three cases the results are far from the conventional figures of 80 to 85 per cent. The

lower as well as the higher conversion ratios in our experiments were not accidental, but resulted from the advised choice of experimental conditions. Former workers, who obtained rather consistently 80 to 85 per cent conversion, did so because they generally used stereotyped, traditional substrate concentrations as well as enzyme preparations that were prepared according to standard recipes.

From the data in Table I it may be noted that in Experiment I the conversion was low simply because the ratio of substrate concentration to enzyme concentration was high (7.5 mg. of starch to 1 mg. of enzyme preparation). In Experiment II, when this proportion was shifted in favor of the enzyme (1 mg. of diastase per 1.5 mg. of starch), the apparent conversion was 105 per cent in 18 hours and reached 111 per cent in 72 hours. In Experiment III the conditions were like those in Experiment II, except that the diastatic activity was further increased by the addition of filtered saliva. As a result, over 100 per cent of the starch was apparently converted to "maltose" after only 12 minutes of incubation, and in 2 hours 122 per cent conversion was shown. These paradoxical figures demonstrate the fallacy of the generally accepted view that the limit of starch conversion is 80 to 85 per cent. They indicate, furthermore, that glucose must constitute a substantial part of the reaction products.

Results of Tripartite Analysis—When the same reaction products were analyzed by the tripartite method, it became evident that such stages, wherever the conventional analytical procedure indicated that the reaction had come to a standstill, actually represented pseudoequilibria, in that significant changes, consisting of shifts between the three groups of reducing substances, were still going on. In Table II are presented the same three experiments as in Table I, but with the reducing matter determined as glucose, maltose, and non-fermentable reducing substances. When the two tables are compared, it may be seen that while in Experiment I in Table I an apparent equilibrium was reached after an 18 hour reaction period, actually, as shown in Experiment I in Table II, no equilibrium had been attained throughout the 72 hours of incubation. It may be noted, furthermore, that instead of a conversion of 51 per cent of the starch to maltose, the aggregate amount of maltose plus glucose in the reaction mixture at no time

exceeded 13.2 per cent of the starch; the bulk of the reduced copper was derived from non-fermentable reducing substances.

TABLE II

Results by Tripartite Analysis

The amounts of copper reduced and the equivalent amounts of sugar are given per liter of reaction mixture.

Experiment No.	Duration of reaction	Copper reduced by non-fermentable reducing matter*	Maltose formed	Starch equivalent of maltose	Glucose formed	Starch equivalent of glucose	Starch converted to maltose and glucose
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
I	1.0	1053	203	193	12	11	6.8
	2.0	1285	263	250	35	32	9.4
	3.0	1350	205	195	139	125	10.7
	18.0	1480	371	352	51	46	13.2
	24.0	1510	307	292	82	74	12.2
	48.0	1570	221	210	128	115	10.8
	72.0	1700	189	180	62	56	7.9
II	0.2	200	175	166	14	13	42.8
	0.4	210	157	149	20	18	40.0
	0.8	200	198	188	7	6	46.3
	1.0	210	166	158	27	24	43.5
	2.0	204	133	126	61	56	43.5
	3.0	169	204	194	33	30	52.3
	18.0	175	200	190	79	71	62.4
	24.0	151	231	220	77	69	69.0
	48.0	155	346	328	6	5	79.5
	72.0	163	222	211	92	83	70.5
III	0.2	45	276	262	90	81	82.0
	0.4	53	276	262	91	82	82.3
	0.8	19	295	280	130	117	95.0
	1.0	21	264	250	152	137	92.8
	2.0	0	290	275	159	143	100.0
	3.0	0	290	275	159	143	100.0
	18.0	64	285	270	127	114	92.0
	24.0	41	301	285	136	122	97.5
	48.0	51	406	386	22	20	97.1

* 1 mg. of glucose ≈ 1.94 mg. of Cu; 1 mg. of maltose ≈ 1.25 mg. of Cu.

In Experiment II an equilibrium was apparently reached with a paradoxical 111.2 per cent conversion of starch to maltose (Table I); actually, however, only 50.5 per cent of the starch was con-

verted to maltose and 20 per cent to glucose (Table II). A large part of the reducing matter again consisted of dextrins.

The results of the tripartite analysis in Experiment III (Table II) furnish added information of interest by showing that when the enzyme concentration is sufficiently high in relation to the substrate concentration a 100 per cent conversion of starch to maltose and glucose, with a complete disappearance of dextrins, is possible. In the experiment cited, this stage was reached in the short period of 2 hours. The fact that at this point glucose constituted one-third of the reaction products accounts for the paradox of a 122 per cent conversion (Experiment III, Table I).

Reversibility of Diastatic Reactions—The figures in Table II reveal that in none of the three experiments presented has an equilibrium been reached even after reaction periods of 2 or 3 days; in fact, the relationship between the three groups of reducing substances has been continuously shifting. This phenomenon, the marked fluctuations in the amounts of the individual reducing substances in the reaction mixture, escapes detection when only the aggregate reducing power of reaction mixtures is determined. In Experiment I, Table II, for example, the amount of maltose decreased in the 3rd hour by about 25 per cent, only to rise to nearly double in the next interval; thereafter it declined consistently, until at the end of 72 hours there was less maltose present than at the end of the 1st hour. Glucose showed similar fluctuations, but the changes in the two sugars bear no quantitative relationship to one another. Close correspondence in the decrease of one to the increase of the other is fortuitous; such relationships do not occur consistently. In the last interval (72 hours), for example, the amounts of both maltose and glucose decreased considerably, glucose twice as much as maltose. Through direct determinations of the actual carbohydrate content of the non-fermentable fractions (by way of hydrolysis and determination of the glucose so formed), it was ascertained that the amounts of the two fermentable sugars that had disappeared reverted to larger, non-fermentable compounds.

The results of Experiments II and III (Table II) exhibit similar characteristics: while at some stages of the reaction the figures are suggestive of interconversion between maltose and glucose, at other stages the changes in the two sugars preclude this possibility.

One experiment of this series is graphically presented in Fig. 1 to illustrate the fact that both maltose and glucose, formed at an early stage of diastatic reactions, may continually and consistently decrease as the reaction progresses; both are shifted back into the non-fermentable fraction.

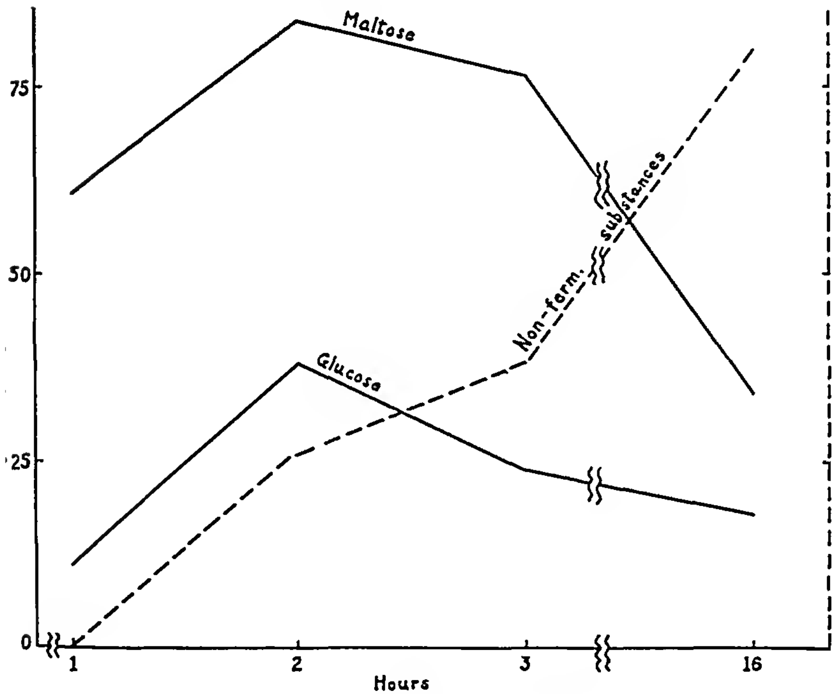


FIG. 1. Example of diastatic reaction in which maltose and glucose decrease, while reducing dextrins increase.

It is indicated, then, that diastatic reactions, between the non-fermentable reducing substances (dextrins) on one side and the two fermentable sugars on the other, are reversible. There is, however, no interconversion between glucose and maltose. We have repeatedly ascertained that the enzyme preparations used in these experiments do not produce glucose when incubated with maltose solutions. In the present study it is shown that when glucose disappears from the reaction mixture it is not converted to

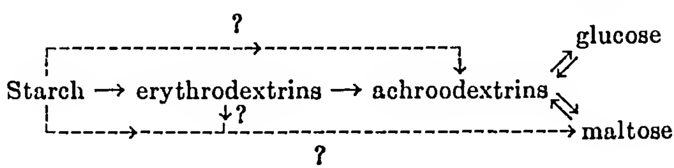
maltose. A good example in proof of this fact may be seen in Experiment III (Table II), in which during the interval between the 3rd and 18th hours of the reaction an appreciable amount of glucose disappeared, without giving rise to an increase in maltose. As was ascertained by hydrolyzing the reaction products by heating with acid, the glucose was polymerized to dextrins (non-fermentable reducing matter). Ling (7) possibly observed such an incidental stage in the action of malt diastase upon potato starch when he reported the conversion of glucose to isomaltose. What Ling regarded as isomaltose was probably a mixture of achroodextrins of rather small molecular sizes, possibly including a non-fermentable disaccharide (3); this is the sort of mixture which in our analyses is denoted as non-fermentable reducing substances.

SUMMARY

The generally accepted view that diastatic reactions reach an equilibrium when 80 to 85 per cent of the substrate (starch) has been converted to maltose is erroneous in more than one respect. The reaction products, instead of consisting of a diastase-resistant grenzdextrin and maltose, represent a mixture of glucose, maltose, and an array of non-fermentable copper-reducing polysaccharides. When the aggregate reducing power of the reaction mixture reaches a virtually constant maximum, *i.e.* when an apparent equilibrium is established, there are still substantial shifts in the amounts of the several reducing substances.

The rate as well as the extent of starch conversion can be readily regulated by proper adjustments of the enzyme to substrate ratio. If the relative amount of the enzyme is high enough, the starch can be completely converted to a mixture of glucose and maltose, but not into maltose alone.

The reactions, dextrins ⇄ glucose and dextrins ⇄ maltose, are reversible, but the conversion of maltose to glucose or the reverse process does not occur in diastatic reactions. Accordingly, Somogyi's diagram representing the course of diastatic action (4) may be expressed in the following amplified form.



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NOTE ON THE FERMENTATION OF MALTOSE AND GLUCOSE IN ALKALINE SOLUTIONS*

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The selective fermentation of glucose in the presence of maltose, in alkaline solutions, furnished the basis of a new approach to the analysis of diastatic reaction products (1). While using this method extensively, we felt that the effect of alkalinity upon the rate of fermentation of maltose deserved additional study from a theoretical as well as a practical point of view.

Fermentation in these experiments was carried out at 30° with washed bakers' yeast, according to the technique described in a previous article (2). The rate of fermentation was measured by the determination of the unfermented sugar in samples periodically withdrawn from the fermenting solution; the fermentation in the samples was stopped by removing the yeast by centrifugation.

The sugar was determined by the copper-iodometric procedure of Shaffer and Somogyi, with the "high alkalinity" reagent (1). Because the sugar solutions contained buffers, precaution was taken to forestall their effect upon the alkalinity of the copper reagent. Failure to consider this effect causes errors in the sugar determination, especially in the acid ranges; the error is as much as 5 to 6 per cent when the sugar solution is buffered at pH 6.0, and about 2.5 per cent even at pH 7.0. This source of error was eliminated by adjusting the buffered sugar solution with sodium hydroxide to about pH 8.0 as described in a subsequent paragraph.

The effect of pH on the rate of fermentation of maltose was studied in the range between pH 3.6 and 8.4. (These figures represent the initial pH values, but in the course of the fermentation they

* This work was aided by the Helen Yonkers Research Fund.

were slightly lowered by carbon dioxide derived partly from the sugar, partly from the "self-fermentation" of the yeast.) From pH 3.6 to 5.2 acetic acid-sodium acetate and from pH 5.2 to 8.0 phosphates were used as buffers; sodium bicarbonate was employed to produce pH 8.4. The concentration of the buffers and of bicarbonate was 0.2 M in all cases.

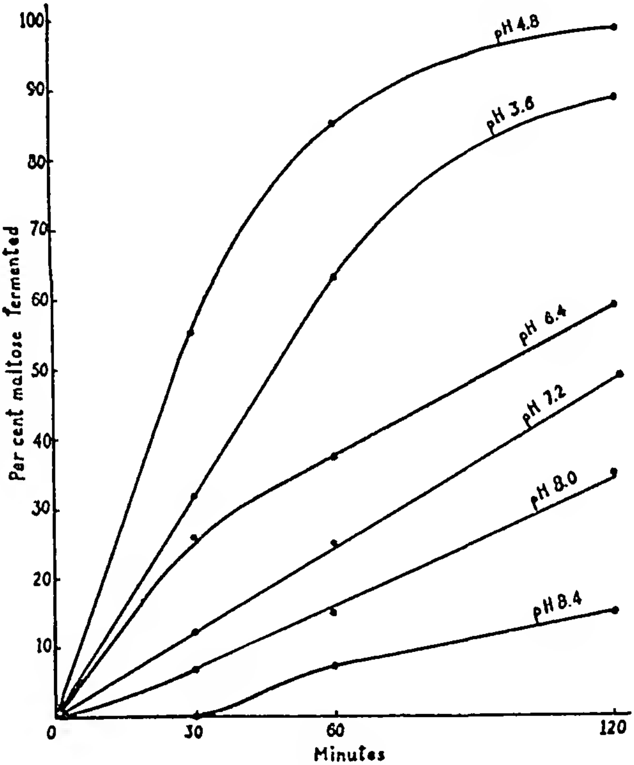

FIG. 1. The effect of pH on the rate of maltose fermentation

In Fig. 1 are represented fermentation rates at a few pH values. As may be seen, the rate was highest at pH 4.8, while at lower as well as at higher pH values fermentation proceeded at lower rates. The optimal pH range, according to data not shown in the graph, lies between 4.8 and 5.2. It may be mentioned that the rate of fermentation of maltose solutions which contain no buffers is distinctly higher than the optimal rate in the presence of buffers, indicating that the electrolytes employed exert a slight inhibitory effect.

Direct Fermentation of Maltose—Willstätter and his collaborators and later Sobotka and Holzman (3) advanced evidence to the effect that maltose is fermented by brewers' yeast directly, without the need of preliminary hydrolysis by maltase. Our results confirm this view in the instance of bakers' yeast. Maltase preparations, extracted from brewers' yeast in our laboratory, showed optimal activity in the pH range of 6.0 to 6.8, much the same as Michaelis and Rona have reported (4). Yet our data show that the time of half fermentation of maltose at pH 6.4, *i.e.* at the optimum of maltase action, was 93 minutes, whereas it was only 27 minutes at pH 4.8, at which maltase activity is almost negligible, and 47 minutes even at pH 3.6, at which maltase is virtually inactive. These figures show that the fermentation of maltose is independent of maltase action.

Application in Analytical Work—Our experiments furnish certain directives for the analysis of diastatic reaction products. When the non-fermentable reducing substances (dextrins) are to be determined, both maltose and glucose must be fermented away. But when diastase is allowed to act in a buffered medium, as a rule at pH 6.8, complete fermentation of the maltose cannot be attained even in 4 or 5 hours, unless either the pH is first lowered to about 5.0 or the phosphates are removed as barium salts. At the optimal pH or in buffer-free solutions, fermentation is complete in 2.0 to 2.5 hours, provided that the yeast employed is fresh. The importance of the latter factor is illustrated in Fig. 2. All three curves show the fermentation rates of unbuffered solutions uniformly containing 40 mg. of maltose per 100 cc., in which 15 gm. (moist weight) of washed bakers' yeast were suspended. In the experiment represented by the topmost curve the yeast was 3 days old (the time necessary to get it from manufacturing plant to laboratory); the other two curves show the marked decrease in the fermentation rate when the same yeast was used 10 and 12 days later.

Selective Fermentation of Glucose—Glucose is readily fermentable at alkaline reactions at which maltose is not affected by bakers' yeast. Inspection of Fig. 1 reveals that at pH 8.4 no measurable amount of maltose is fermented in 30 minutes with as much as 15 gm. of yeast suspended in 100 cc. of solution. Table I shows that at the same alkalinity glucose is almost completely fermented

in 10 minutes with only 10 gm. of yeast per 100 cc. of solution. These facts form the basis of the quantitative determination of glucose in the presence of maltose.

In our earlier studies of diastatic reactions we used no buffers. For selective fermentation the reaction products were made alkaline by the addition of sodium carbonate, and a 30 minute period was allowed for the complete fermentation of glucose. During

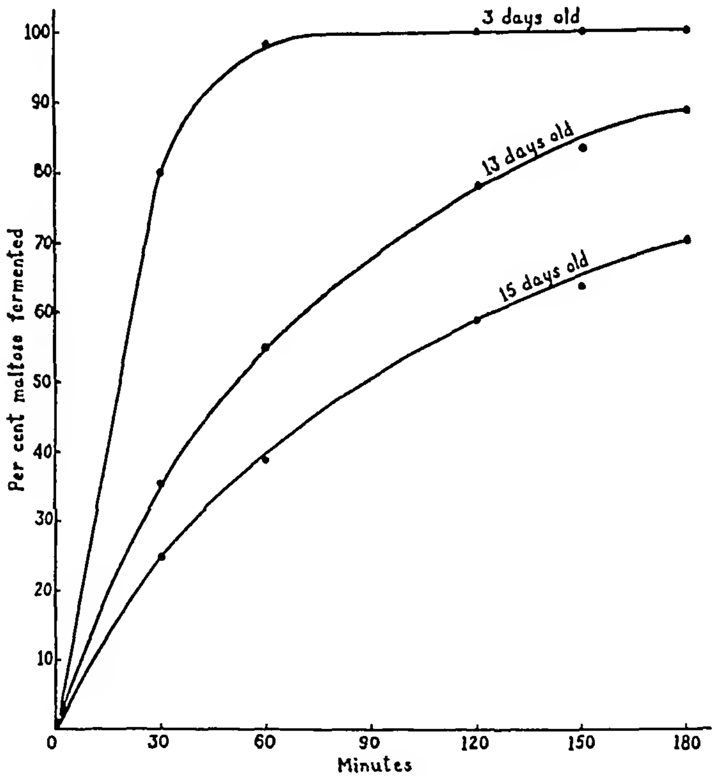

Fig. 2. The effect of age of yeast on the rate of maltose fermentation

these 30 minutes it was necessary to watch the reaction of the fermenting fluid lest the pH drop owing to the evolution of carbon dioxide partly from the sugar, partly from the "self-fermentation" of yeast. As may be seen in Fig. 1, this was important, since at pH 8.0 a measurable fraction of the maltose also is fermented in 30 minutes. In the present studies, in which phosphate buffers of 0.2 M concentration were used, we found that

adjustment of the pH to 8.4 is sufficient to safeguard the sharp separation between glucose and maltose.

Tripartite Analysis—Accordingly, when glucose, maltose, and reducing dextrins are to be determined in buffered reaction mixtures, we proceed as follows: A 20 cc. portion of the reaction mixture is measured, a drop of phenolphthalein added, and then, from a burette, sodium hydroxide is run in until a pale but permanent pink color is attained; finally the solution is diluted to 25 cc. or to some other convenient volume. Two 5 cc. portions of this alkalinized solution are used for the determination of the aggregate reducing power (A). The remainder is fermented with washed yeast, approximately 10 gm. (moist weight) being used per 100 cc. of fluid. After about 20 minutes fermentation the

TABLE I

Rate of Glucose Fermentation at pH 8.4

Fermentation time	Unfermented fraction of glucose		
	With 5 per cent yeast	With 10 per cent yeast	With 15 per cent yeast
min.	*mg. per cent*	*mg. per cent*	*mg. per cent*
0	51.2	51.2	51.2
10	24.8	0.4	0.5
20	11.4	0	0
30	5.7	0	0

yeast is removed by centrifugation, and the reducing power (B) of the solution is determined. $A-B=$ glucose.

To determine the reducing power of the non-fermentable reaction products (dextrins), both glucose and maltose must be completely fermented away. For this fermentation the reaction of the solution must be adjusted to a pH between 4.5 and 5.2 by the addition of hydrochloric acid. By titrating 10 cc. of the reaction mixture with the acid, with methyl red as an indicator, the amount of acid required is determined. On this basis 20 cc. of the reaction mixture are acidified to the optimal pH of maltose fermentation, the volume is brought to 25 cc., and then the mixture is fermented with 15 per cent washed yeast for 2.5 hours. After the yeast is removed by centrifugation, 5 cc. portions of the fluid are measured into sugar tubes for the determination

of the reducing power. Before the copper reagent is added, the reaction of the solution is adjusted to pH 8.0 to 8.4 by adding sodium hydroxide, with penolphthalein or phenol red as indicator. The reducing power (C) of this solution represents non-fermentable substances. $B - C =$ maltose.

SUMMARY

Maltose is fermented by bakers' yeast without the need of preliminary hydrolysis to glucose by the action of maltase ("direct fermentation").

At pH 8.4 glucose is completely fermented in less than 20 minutes, whereas under the same conditions no measurable amount of maltose is fermented in 30 minutes. This fact is employed as the basis of an analytical procedure for the determination of glucose in solutions which contain maltose.

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A STUDY OF THE ACTION OF PANCREATIC AMYLASE*

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Studies of the inactivation of biologically active proteins by chemical reagents are assuming new significance. This is due to the fact that many compounds are now known which act in definite and specific ways upon certain groupings in the protein molecule. While the characteristic activities of these proteins must be considered primarily a property of the molecule as a whole, there is no doubt that in many cases they also depend upon the presence and the arrangement of certain chemical groups in the molecule. This being the case, the blocking or destruction of such groups by specific reagents, paralleled by a study of their effect upon the activity, has proved to be most informative.

The three groups which have so far been found to be closely connected with the specific activities of such proteins are the free primary amino groups (1–4), which are probably those of lysine, the sulfhydryl groups of cysteine (5–12), and the phenolic hydroxyl groups of tyrosine (13–17).

This investigation was undertaken to determine, if possible, whether any of these groups is responsible for the activities of pancreatic amylase. The value of such work with regard to enzymes is obvious, as it adds precise information about the nature of the enzyme and the way in which it acts. Pancreatic amylase is especially well suited to such an investigation, as it has been shown (18, 19) to be protein in nature and is rather easily purified, although the yields are small (18). Much of the work reported here was carried out with the highly purified amylase (18). In

* This work is taken from a dissertation submitted by John E. Little in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

some cases the more stable and readily available pancreatin[1] was also used. The results with the amylase from the two sources are in accord, as would be expected from the fact that the amylase activity is always taken as the direct measure of the concentration of active enzyme present.

EXPERIMENTAL

Acetylation—Ketene was used to acetylate the amylase. This reagent has been employed successfully by Northrop and Herriott in their work with pepsin (15–17), by Stern and White in their work with insulin (20), and by a number of other investigators (2, 3, 21). Ketene reacts with primary amines, sulfhydryl groups, and phenolic tyrosine groups, but not with secondary amines, or aliphatic hydroxyl groups (13, 22–24). Moreover, its reaction with primary amines is much more rapid than that with either the sulfhydryl or the phenolic groups (13, 15). This point is of special interest in connection with the work to be reported here.

The effect of acetylation upon pancreatic amylase was studied by following both its saccharogenic (25) and its amyloclastic (26) activities. To make certain that the effects observed were due to the acetylation and not to the other conditions of the experiments, the activities of treated solutions were always compared side by side with the activities of aliquots of the original unacetylated enzyme solution which had been held under the same conditions as the acetylated portions.

The acetylations were carried out at 0° in aqueous solutions at pH 5.0 in the presence of 2 M acetate, 0.01 M phosphate, and 0.02 M sodium chloride. The phosphate and chloride were added to aid in the stability of the enzyme (18). A few drops of caprylic alcohol were also always added to prevent foaming which has been found to decrease the activity of the protein (18). A modification of the apparatus described by Li (27) was used.

Results

Table I gives typical data for the rate of inactivation of the saccharogenic activity of the amylase when it was acetylated with ketene for 150 minutes. Aliquots of the acetylated solution were

[1] We wish to thank Parke, Davis and Company for the highly active undiluted pancreatin they so kindly supplied us for this work.

removed at the intervals noted and kept in melting ice until the acetylation of the rest of the solution was completed. They were then measured, together with the control solution, for saccharogenic activity (25, 28). For this they reacted for 30 minutes at 40° with 1 per cent soluble starch at pH 7.1 and 2 cc. of the resulting reaction mixtures were tested for reducing value (25) which was calculated in terms of maltose (29). The data are readily reproducible provided the rate of ketene flow is held constant.

The rapid decrease in saccharogenic activity (69 per cent in 30 minutes) indicates that the loss is due to the acetylation of amino

Table I

Action of Ketene upon Saccharogenic Activity of Pancreatic Amylase

Acetylation	Maltose per 2 cc. reaction mixture	Activity*	Activity in per cent of original
min.	*mg.*		
0	7.51	751	100.0
30	2.33	233	31.0
60	1.55	155	20.6
90	0.86	86	10.1
120	0.69	69	9.2
150	0.52	52	6.9
150 (Control)	7.25	725	96.5

* Activity = mg. of maltose per mg. of enzyme under stated conditions which include that the measurements be made upon the linear portion of the activity curve (25).

groups rather than to that of phenolic groups of tyrosine or of sulfhydryl groups of cysteine, which presumably would both have been acetylated much more slowly (1–3, 13, 15, 20). This conclusion was confirmed by all subsequent work.

The data given in Fig. 1 show a close correlation between the loss of amylase activity and the loss of amino nitrogen due to the action of the ketene. The loss of amino nitrogen was determined according to Van Slyke (30) with a micro apparatus and the technique was standardized and checked by analyses of solutions of alanine containing known amounts of amino nitrogen.

It is interesting to note that the rate of loss of amylase activity along the first part of the curve is somewhat more rapid than the

rate of the blocking of the amino nitrogen and that the curves approach each other as the losses of activity and of amino nitrogen become complete. Fig. 2 illustrates this more clearly. Extrapolating the curve as shown by the dotted line indicates that 100

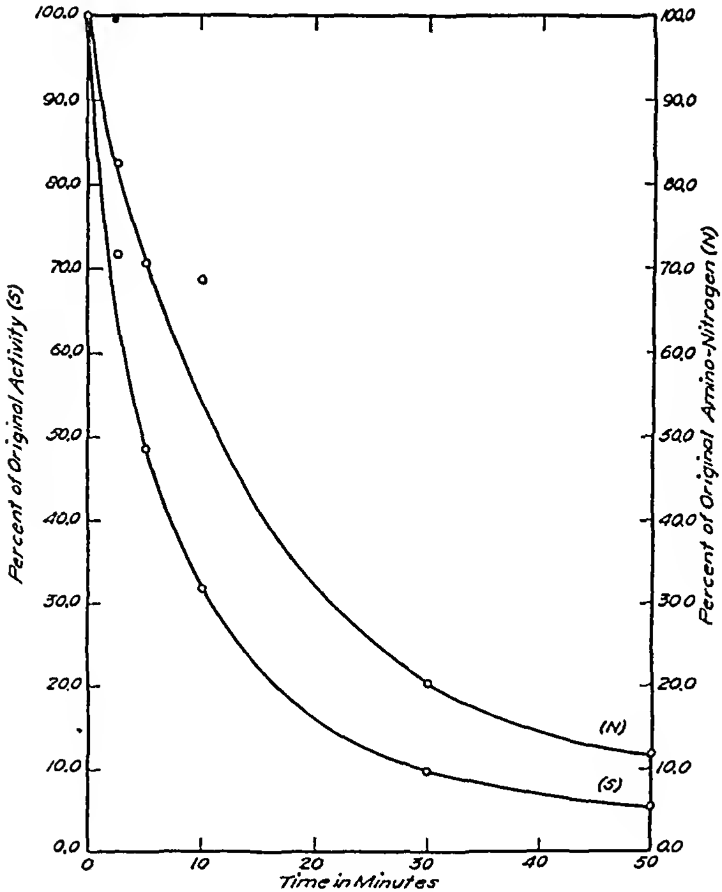

FIG. 1. Comparison of the losses of the saccharogenic activity (S) and of the amino nitrogen (N) of pancreatic amylase with time of acetylation during treatment with ketene. The data are given in per cent of original activity or of original amino nitrogen.

per cent inactivation would be realized at the point where the blocking of the amino groups would be complete. All of the amino groups must, therefore, be in some degree necessary to the enzymic action. The larger initial rate of loss of activity may mean that the amino groups which are more accessible to, or re-

active with ketene are also those most active in the sugar-forming action of the enzyme, and, therefore, that the blocking of these groups would cause a greater drop in activity than that of those acetylated with more difficulty later.

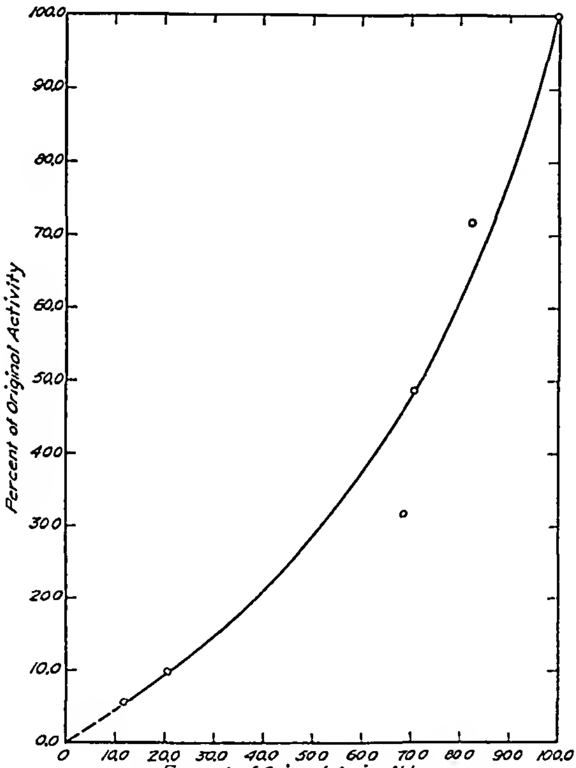

Fig. 2. Loss of the saccharogenic activity of pancreatic amylase with loss of its amino nitrogen upon acetylation with ketene. The data are given in per cent of original activity or of original amino nitrogen.

In Fig. 3 a comparison is made of the rates at which the amylase loses its amyloclastic and saccharogenic activities upon acetylation with ketene. The data were obtained when aliquots of the same enzyme solution, before and after acetylation, were measured for both kinds of activity and are typical of a number of such comparisons. They show that the amyloclastic activity is lost

at even a slightly more rapid rate than the saccharogenic activity and indicate that the former as well as the latter depends upon free amino groups in the enzyme molecule but perhaps not entirely upon the same ones. If the losses of the two activities were due

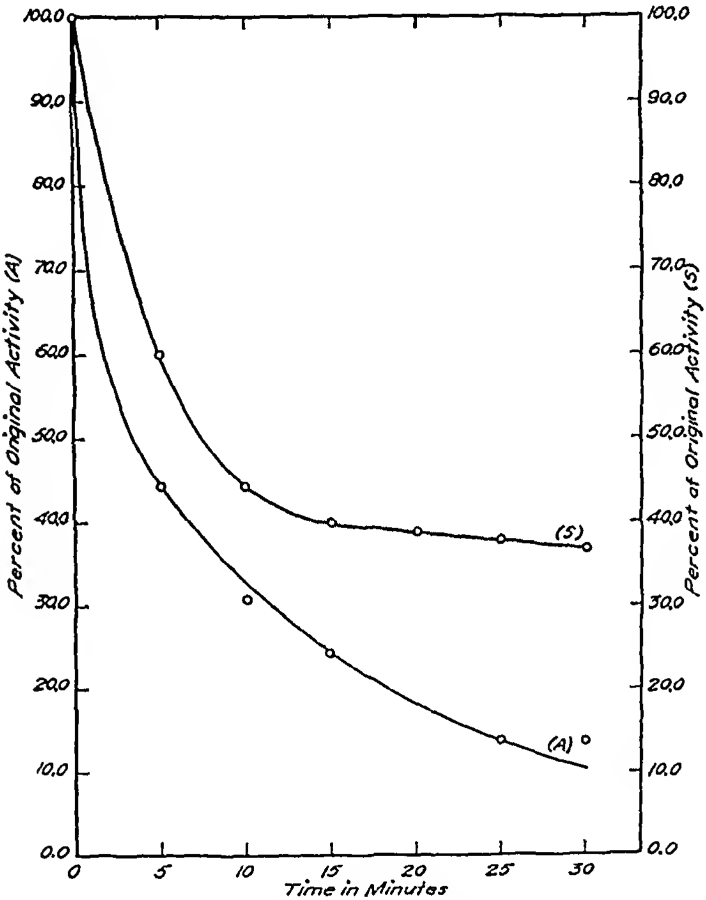

FIG. 3. Comparison of the losses of the saccharogenic (S) and of the amyloclastic (A) activities of pancreatic amylase with time of acetylation during treatment with ketene. The data are given in per cent of original activities.

to the acetylation of different kinds of groupings, such as the phenolic hydroxyl groups of tyrosine or the sulfhydryl groups of cysteine in one case, and the primary amino groups of lysine in the other, the rates of loss of the two activities would presumably be very different, as the former groups have been reported by a

number of workers (1–3, 13, 15, 20) to be acetylated in proteins much more slowly than the primary amino groups.

The relatively small differences observed in the rates of loss of the two types of activity may be due to differences in reactivity or availability of essential amino groups to ketene, depending upon the positions of these groups in the protein molecule.

While the above data lead to the conclusion that the activities of pancreatic amylase are intimately connected with its primary amino groups, it seemed of interest to study more definitely the possible importance of the phenol groups of its tyrosine, especially as such groups have been found to be closely related to the activities of pepsin (15–17) and of other biologically active proteins (2, 3, 21).

A solution of the amylase was acetylated in the usual manner and aliquots of the solution before and after treatment were measured for free phenol groups of tyrosine by the "pH 8" method of Herriott (16). It was found that 15 minutes acetylation, which is sufficient to cause a loss of 78 per cent of the activity of the amylase and a loss of 40 per cent of its free amino nitrogen, had caused only a 13 per cent loss of the free tyrosine groups of the protein. These results, taken with the finding that the activity of the amylase disappears entirely only when its amino groups are completely blocked, tend to eliminate the tyrosine groups as an important factor in the activity of this amylase.

This conclusion was further strengthened by the fact that no activity was recovered when inactivated acetylated material was subjected to mild hydrolysis at pH 11 under conditions which have been found suitable for the removal of the acetyl groups from acetylated pepsin (15, 16) and from acetylated insulin (31). Completely acetylated, inactive material remained inactive after the hydrolysis. Partially inactivated acetylated material did not increase in activity but also did not lose its partial activity. This showed that the hydrolysis treatment itself was not too drastic, had not appreciably disrupted the peptide linkages of the protein molecule.

The conditions for the hydrolysis of acetylated phenolic groups as given by Herriott and Northrop (15, 16) are without effect upon the acetylated amino groups of the protein, as these are, in general, much more difficult to hydrolyze than the acetylated tyrosine

groups (15, 16, 32). Hydrolysis of the partially acetylated material with acid or with papain resulted in the further loss of all of its activity, presumably because of destruction of the protein molecule.

Other Reagents—Only brief mention will be made of a number of other experiments which give additional direct evidence that the activity of pancreatic amylase is intimately connected with the presence of free primary amino groups in the protein molecule. A number of reagents which react specifically with primary amino groups of proteins were studied.

The amylase was completely inactivated by treatment with formaldehyde, while the control solutions, kept under identical conditions, except for the formaldehyde, showed no loss of activity. The formaldehyde reacted with the enzyme for 30 minutes at 25° in 1 per cent concentration and at pH 8.2 (2, 33–37).

Treatment of pancreatic amylase with phenyl isocyanate at pH 7.1 for 5 minutes (36, 38–40) caused a 73 per cent loss in its saccharogenic activity, while the control solution, without the isocyanate, retained its full activity.

Nitrous acid (14) also caused rapid and complete loss of pancreatic amylase activity. The detailed results of this work are of special interest and are being reported elsewhere.[2]

Although the experience of this laboratory indicates that pancreatic amylase is not easily oxidized or reduced and neither the highly purified amylase nor the pancreatin gave evidence of the presence of sulfhydryl groups, detectable by the nitroprusside test, it seemed of interest to make a quantitative study of the effect of mild oxidation (aeration) and reduction (treatment with cysteine) upon the amylase activities.

In the aeration experiments, air was bubbled through a solution of the amylase at pH 5.0 and 0°. The bubbling was at the rate of about two bubbles a second and a few drops of caprylic alcohol were present to prevent foaming (18). When the pancreatic amylase was treated with cysteine, the highly purified enzyme was allowed to stand in contact with 0.05 M cysteine for 30 minutes at pH 7.1 at 0°. Neither of these treatments had any appreciable influence upon either the saccharogenic or the amyloclastic activity of the amylase.

[2] Little, J. E., and Caldwell, M. L., unpublished data.

The effect of iodoacetic acid which, under certain conditions (41), is specific for sulfhydryl groups of proteins was also studied and found to be in strong contrast to the effect of the reagents discussed above which react specifically with primary amino groups and which cause prompt inactivation of the amylase. No loss of activity could be detected when highly purified pancreatic amylase was treated with 0.1 M iodoacetic acid at pH 7.1 for 30 minutes at 0°. Such treatment causes inactivation of urease (12) and of a number of other enzymes and proteins (7, 11) which are, therefore, believed to owe their characteristic activities in part at least to the presence of free sulfhydryl groups.

Taken together, the results of these latter experiments as well as those of the acetylation show quite conclusively that neither sulfhydryl nor disulfide groups are essential to the activity of pancreatic amylase.

SUMMARY

A study of the influence of acetylation, with ketene, and of the action of certain other reagents upon the activities of pancreatic amylase has been made.

It has been established that the primary amino groups of the enzyme protein are essential to the activities of this amylase.

It has been shown that the phenolic hydroxyl groups of tyrosine are of little, if any, importance to the activities of this amylase.

No evidence has been found for the presence of free sulfhydryl groups in the active enzyme nor for their importance to its activities.

At any given time during the acetylation of the enzyme with the ketene, the per cent loss of the amyloclastic activity is slightly greater than the per cent loss of the saccharogenic activity. This seems to indicate that the amino groups responsible for the two kinds of activity may not always be identical but that there may be a difference in reactivity and availability to ketene between those amino groups most essential to the amyloclastic activity and those most necessary to the saccharogenic activity of the enzyme.

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THE EFFECT OF DIBENZANTHRACENE, OF ALCOHOL, AND OF OTHER AGENTS ON VITAMIN A IN THE RAT*

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Dibenzanthracene tends to minimize hepatic stores of vitamin A (1, 2) both by inhibiting the accumulation of the vitamin and by accelerating the rate of depletion of preformed stores (3). These effects could be the result of various mechanisms. The hydrocarbon might promote the excretion of the vitamin; it might hasten the destruction of the vitamin in the body; or it might alter the distribution of vitamin A between the liver and other organs. The first possibility was considered by Goerner and Goerner (1) who, however, failed to find any vitamin A in the urine of their injected animals. We have been unable to detect the vitamin in the combined excreta. The second possibility does not readily lend itself to experimentation, and accordingly we have examined the third. The effects of the hydrocarbon were compared with other regimens likely to affect vitamin A storage, and an attempt was made to determine the fate of that fraction of the vitamin which had been driven from the liver by the dibenzanthracene.

Methods

Essentially the same methods were employed as in our previous studies (3). Vitamin A stores were established in standard rats

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by the ingestion of halibut liver oil, and the rats were then continued on various modifications of a standard diet low in vitamin A. When changes in the protein content of this ration were introduced, corresponding alterations were made in the carbohydrate component, cooked corn-starch. Colloidal 1,2,5,6-dibenzanthracene was prepared and injected as before and the amount of vitamin A remaining in the various tissues of the animals was determined chemically by the $SbCl_3$ method. The results, initially obtained as blue units, were expressed as micrograms of vitamin A per tissue. 5 blue units = 1 γ (4).

EXPERIMENTAL

Effect of Dibenzanthracene on Distribution of Vitamin A in Rat Tissue—Twenty-six rats, 36 to 48 gm. in weight, were given 6 drops of halibut oil equivalent to a total of 1560 γ of vitamin A. Two animals were killed for analysis and the remainder placed on various diets free of the vitamin. Certain groups were injected intraperitoneally with 1 mg. of colloidal dibenzanthracene in 1 cc. of 1 per cent gelatin weekly. After 8 weeks all animals were killed, and the livers as well as the combined non-hepatic organs (lungs, kidneys, adrenals, spleen, and 4 cc. of blood) were analyzed for vitamin A. Attempts to determine the vitamin in the entire non-hepatic carcass were unsuccessful.

As in our previous studies (3) the animals injected with dibenzanthracene contained significantly less hepatic vitamin A than the uninjected animals, and this was observed consistently in spite of other variables introduced. With the decrease of vitamin A in the liver, there was a definite increase of the vitamin A in the non-hepatic tissues of all injected animals. This increase was particularly evident when the percentage distribution of vitamin A between the liver and the non-hepatic tissues was calculated. In the absence of dibenzanthracene 2 per cent or less of the total vitamin in the body existed outside the liver; in rats treated with dibenzanthracene, 19 per cent or more of the total vitamin was found to be in the non-hepatic organs (see also Table IV). When the experiment was repeated with somewhat heavier animals, and the various non-hepatic organs analyzed separately, the non-hepatic vitamin A was found to be almost entirely in the kidneys (Table I).

Numerous variations in experimental procedure involving 130 rats were introduced in an attempt to determine the extent and limitation of this translocation of vitamin A. Beginning with initial stores of 750 γ of vitamin A per liver, a marked shift to the kidney was always observed when the animals were exposed to the hydrocarbon for a sufficient length of time to reduce the hepatic stores to one-fifth of their original value, or 150 γ. This required a depletion period of 8 weeks. With a longer time of contact the amount of vitamin A in the kidney increased until in certain animals it actually exceeded the amount in the liver. On the other hand, when the animal was exposed to dibenzanthracene for 7 weeks or less, and the hepatic stores reduced to one-third of their original value, or 250 γ, only a relatively slight diversion of vitamin A to the kidneys was observed.

TABLE I

Effect of Dibenzanthracene on Distribution of Vitamin A between Liver and Kidney

No. of rats	Time on experiment	DBA* injected	Vitamin A in liver	Vitamin A in kidneys	Vitamin A in lungs, etc.	Body vitamin A in kidneys
	wks.		*γ per rat*	*γ per rat*	*γ per rat*	*per cent*
2	0	−	869 (858–880)	18†		2.0
3	9	−	685 (456–946)	10	2	0.3
3	9	+	128 (64–252)	43	0	25.2
2	0	−	880 (840–920)	20	4	2.2
4	8	−	506 (386–648)	2	1	0.4
4	8	+	132 (61–210)	23	1	15.0

* 1,2,5,6-Dibenzanthracene.

† Combined non-hepatic organs.

The diversion to the kidney was also slight when storage of vitamin A proceeded under the influence of the hydrocarbon. A group of eight rats, weighing 69 to 105 gm. and containing less than 20 γ of vitamin A per liver, was fed the standard low vitamin A diet and injected intraperitoneally 3 times during 1 week with 1 mg. of colloidal dibenzanthracene in 1 cc. of 1 per cent gelatin solution. A similar group was injected with gelatin only. All rats were then fed 4 drops of halibut liver oil and were killed for analysis 3 days later. The control animals averaged 616 γ of vitamin A per liver (530 to 840) and 6.5 γ per pair of kidneys; those receiving dibenz-

anthracene averaged 380 γ per liver (209 to 540) and 15 γ per pair of kidneys. In other words, the kidneys of the control animals contained 1.0 per cent of the total body vitamin A as compared to 3.8 per cent in the rats treated with dibenzanthracene.

Even under conditions favorable for a vitamin A shift, the increase of the vitamin in the kidney accounted for only a small fraction of that which had disappeared from the liver. A typical case is presented in Table II. Under the influence of the dibenzanthracene the vitamin A content of the kidney increased from 5 to 69 γ in 9 weeks, but during the same period 605 γ of vitamin A were lost from the liver. In other words, only 10.5 per cent of the vitamin, which had left the liver, reappeared in the kidney. Incidentally dibenzanthracene promoted a vitamin A shift, whether injected subcutaneously in oil (Table II) or intraperitoneally as a gelatin emulsion (Tables I, IV). The shift was also observed in four rats injected with methylcholanthrene.

TABLE II

Percentage of Mobilized Vitamin A Diverted to Kidney

	Control rats, no DBA,* 0 wk.	Control rats, no DBA,* 9 wks.	1 mg. DBA* per wk. subcutaneously in oil, 9 wks.
	γ	γ	γ
Vitamin A in liver	783	496	178
" " " kidney	5	Trace	69
Total vitamin A	788	496	247
Vitamin A lost from liver		287	605
Increase of vitamin A in kidney		0	64
% of lost vitamin A in kidney		0	10.5

* 1,2,5,6-Dibenzanthracene.

In the shift of vitamin A from the liver to the kidney, the hydrocarbons have apparently upset the equilibrium which ordinarily exists between hepatic and renal vitamin A. In the normal animal this distribution is overwhelmingly in favor of the liver (5, 6) and even the high amounts of vitamin which must have been present in the blood after the ingestion of halibut liver oil nevertheless failed to produce any major accumulation of vitamin A in the kidneys (initial controls, Tables I and II). After 9 weeks on a low vitamin A diet the hepatic stores of the vitamin were reduced

by less than 50 per cent and the renal stores were also reduced, often to amounts which were no longer measurable (Tables I and II). This confirms older observations on a general parallelism between changes in the vitamin A content of liver and kidney (6, 7). The parallelism also persisted in eight uninjected rats which were continued on our low vitamin A diet for 18 and 24 weeks. Hepatic stores decreased from 734 γ to 399 and 99 γ of vitamin A respectively, levels roughly comparable to those after only 8 weeks in contact with dibenzanthracene. Only traces of vitamin A remained in the kidneys. In other words the mere removal of large amounts of vitamin from the liver failed to increase the vitamin content of the kidney. Under the influence of dibenzanthracene, however, the vitamin content of the kidney increased, while the total amount in the body was decreasing.

A cumulative effect of the hydrocarbon was indicated by the fact that the vitamin A shift was most pronounced in the long time experiments. Dibenzanthracene is somewhat soluble in body fluids (8, 9) and it is known to wander from the site of injection to other areas, particularly to the lungs (10, 11) and liver (12, 13), where various deleterious effects result. The administration of hydrocarbons frequently results in the appearance of fluorescent substances in the liver (14) or bile (15, 16). Accordingly, it is suggested that dibenzanthracene may act by interfering with the firmness of combination between vitamin A and the unknown to which it is normally attached in the liver, presumably protein; or, in other words, that dibenzanthracene may alter the equilibrium between free and bound vitamin A in favor of the free form, which can then move away to tissues where it is more readily destroyed. Destruction of the mobilized vitamin seems probable, since only 10 per cent of it reappears in the kidney.

Effect of Alcohol on Vitamin A Storage—Of the various substances which have been reported to alter vitamin A storage, ethyl alcohol seems to resemble dibenzanthracene most closely in its effect on the vitamin. Alcohol has been reported to decrease the storage of vitamin A in the livers of guinea pigs (17), and to increase the vitamin A content of the blood in dogs (18) and in humans (19). Visual adaptation to dim light may also be improved by alcohol (20). Taken collectively, the results obtained in the various species indicate a shift of vitamin from the liver to the blood, *viz.*,

an altered distribution of vitamin A. Accordingly it was of interest to study alcohol and dibenzanthracene under comparable conditions.

Thirty-nine young rats were fed halibut liver oil until hepatic stores of 678 to 869 γ were attained. They were then divided into three groups and fed the low vitamin A diet for from 7 to 14 weeks. Group 1 received no additional treatment, Group 2 received a 15 per cent solution of alcohol in place of drinking water, while Group 3 drank water but received 1 mg. of colloidal dibenzanthracene per week intraperitoneally. Alcohol invariably hastened the depletion

Table III

Effect of Alcohol on Hepatic Vitamin A

Series No.	Initial stores	Hepatic storage after depletion on low vitamin A diet			Length of experiment
		No alcohol	15 per cent alcohol	1 mg. DBA* per wk.	
	γ per liver	*γ per liver*	*γ per liver*	*γ per liver*	*wks.*
XI	678	478	263 (246–282)	183	8
XII	783	650	286 (122–406)	233	8
XVII	869	858	716	255	7
"	869	685	306	128	9
XX	734	>403	114 (19–231)		10
"	734	403	53 (0–104)		14
Average........	778	595	290	<200	
No. of rats.....	8	13	15	11	

* 1,2,5,6-Dibenzanthracene.

of hepatic vitamin A, although only about one-half as effectively as dibenzanthracene (Table III). In contrast to the hydrocarbon, alcohol did not promote a detectable shift of vitamin A from the liver to the kidney. There was a slight but irregular increase of vitamin A in the combined non-hepatic organs of the rats fed alcohol, and some indication of an increase in the lung, but in general the amounts of vitamin present were too small for accurate measurement. Thus the effect of the alcohol was neither quantitatively nor qualitatively identical with that of dibenzanthracene.

Effects of Miscellaneous Substances on Hepatic Vitamin A—Vitamin E, *l*-cystine, bromobenzene, and anthracene were each

studied as possible modifiers of the dibenzanthracene effect. In the first series fifteen young rats containing 783 γ of vitamin A per liver were divided into five groups and fed various rations free of vitamin A. Group 1 was placed on our ordinary low vitamin A control ration (3) which contains vitamin E as 5 per cent cottonseed oil. Group 2 was fed the control ration minus the cottonseed oil. This diet was free of vitamin E and incidentally also low in the essential fatty acids. Group 3 received a high vitamin E diet: 25 mg. of α-tocopherol per kilo of control ration. Groups 4 and 5 were fed the control and the high vitamin E diets respectively and in addition were injected intraperitoneally with 1 mg. of colloidal dibenzanthracene per week. After 8 weeks all animals were killed for analysis.

Vitamin A depletion was somewhat more rapid in the complete absence of vitamin E than in its presence. On the low vitamin E diet 617 γ of vitamin A remained per liver after 8 weeks as compared to 650 γ on the diet containing cottonseed oil, thus essentially confirming Moore (21, 22). However, additional amounts of vitamin E did not further retard the depletion of vitamin A, nor did vitamin E counteract the effect of dibenzanthracene. Only 226 γ of vitamin A remained in the livers of rats treated with dibenzanthracene, whether excess vitamin E was present or not. In other words, the effect of the hydrocarbon was quantitatively much greater than that of vitamin E depletion.

An effect of cystine on vitamin A storage was sought in an analogous way. The various diets fed and the results obtained are summarized in Table IV. In no case did cystine materially alter the effectiveness of the dibenzanthracene, unless possibly when it was added at a 0.5 per cent level to a diet containing 12 per cent of casein, and even in this particular group the injected animals contained relatively low amounts of vitamin A, 182 γ as compared with the 381 and 478 γ remaining in animals not treated with dibenzanthracene. Thus, although cystine nullifies the effect of hydrocarbon on the growth rate of young animals (23), it did not nullify the effect on hepatic vitamin A. Two substances which increase the cystine requirement of rats, bromobenzene (24) at 0.2 per cent and anthracene (25)[1] at 0.1 per cent, likewise failed to alter the rate of vitamin A depletion.

[1] DeLeers, V. J., and Baumann, C. A., unpublished data.

Dietary Protein and Hepatic Vitamin A—In a previous series (Table IV) it was noted that the depletion of hepatic vitamin A was more rapid when the casein content of the diet was reduced from 18 to 12 per cent. The effect of protein level was therefore studied in some detail. Young rats containing 311 to 869 γ of vitamin A were fed low vitamin A diets containing 8 or 10 per cent of casein for periods of 5 to 9 weeks. Comparable animals received the standard low vitamin A diet containing 18 per cent of casein.

The livers of rats fed the low protein diets almost invariably contained less vitamin A than the livers of animals fed the higher amounts of protein (Table V). The single exception (Table V, Series XII) was a group of rats which had been injected with dibenzanthracene, and apparently the rate of depletion of the vitamin in this group was already sufficiently rapid under the influence of the hydrocarbon so that the low level of protein exerted no additional effect.

TABLE IV

Effect of Cystine and of Dibenzanthracene on Vitamin A Distribution

Low vitamin A diets	DBA* injected	No. of rats	Time on experiment	Vitamin A per liver	Vitamin A in non-hepatic organs	Total vitamin in non-hepatics
			wks.	γ	γ	*per cent*
"Control" (18% casein)	−	2	0	678 (651–704)		
18% casein	−	4	8	478 (341–564)	4 (Pooled)	1
12% "	−	3	8	381 (336–452)	8 (5–13)	2
18% "	+	4	8	183 (104–330)	43 (41–44)	19
18% " + 0.5% cystine	+	4	8	149 (46–316)	36 (12–66)	19
12% casein	+	3	8	122 (34–216)	58 (29–72)	32
12% " + 0.5% cystine	+	3	8	182 (171–216)	43 (13–58)	19

* 1,2,5,6-Dibenzanthracene.

Parallel experiments were performed on the formation of hepatic stores of vitamin A. Low vitamin A diets of high and low protein content were fed to young rats for 2 weeks, after which they were given 3 or 4 drops of halibut liver oil and killed for analysis 2 days later. The dietary alterations and the results are listed in Table VI. A preliminary feeding of diets containing 8 per cent of

Table V

Effect of Diets Low in Protein on Vitamin A Depletion

Series No	Length of experiment	DBA* injected	Casein in low vitamin A diet	Rats per group	Vitamin A per liver: Low protein diet	Vitamin A per liver: 18 Per cent casein diet	Vitamin A per liver: Initial
	wks.		*per cent*		γ	γ	γ
XI	8	−	12	3	381 (336–452)	478	678
"	8	+	12	3	122 (34–216)	183	678
XII	7–9	−	10	2	462 (444–484)	650	783
"	7–9	+	10	3	236 (164–369)	233	783
XVI	5–6	−	10	5	97 (18–142)	200	311
"	5–6	+	10	4	2 (0–9)	19	311
XVII	7	−	8	1	612	858	869
"	9	−	8	3	665 (587–714)	685	869
XX	8	−	8	2	440 (334–546)	463	
XXI	8½	−	8	3	291 (134–328)	379	648

* 1,2,5,6-Dibenzanthracene.

Table VI

Effect of Preliminary Low Protein Diets on Entry of Vitamin A into the Liver

Series No.	Dietary treatment during preliminary period*	Weight change in 2 wks	No. of rats	Halibut oil fed	Vitamin A per liver
		gm.		*drops*	γ
XVIII	18% casein *ad libitum*	75–121	4	4	1021
"	8% " " "	73–102	4	4	629
XIX	18% " " "	101–137	3	4	1363
"	36% " " "	99–134	3	4	1314
"	8% " " "	97–121	3	4	636
"	8% " + 12% gelatin *ad libitum*	89–95	3	4	457
XXII	18% casein *ad libitum*	66–79	2	3	696
"	18% " " " †	55–50	2	3	368
"	8% " " "	60–70	4	3	430
XXIII	8% " " "	39–66	4	3	288
"	18% " " "	40–102	4	3	383
"	18% " restricted	38–69	4	3	345
"	8% " + 12% gelatin *ad libitum*	40–62	4	3	220
XXVI	8% casein *ad libitum*	40–70	4	4	201
"	18% " food intake restricted 33%	38–70	4	4	268

* Low vitamin A diets fed for 2 weeks.

† Animals appeared visibly sick after 2 weeks.

casein reduced the subsequent storage of vitamin A by 40 to 50 per cent, although the weights of the animals were only 15 to 20 per cent under those fed 18 per cent of casein. In Series XXIII and XXVI, Table VI, the food intake of rats fed 18 per cent casein was so restricted that growth did not exceed that of animals fed 8 per cent of casein *ad libitum*. The restricted animals on the higher percentage of casein invariably had the smaller livers, 3.3 gm. as compared to 3.9 gm. average; yet they developed larger stores of vitamin A. Apparently, therefore, low protein *per se* reduced vitamin A storage. However, storage was no greater on 36 per cent of casein than on 18 per cent, and the addition of 12 per cent of gelatin to 8 per cent of casein failed to increase vitamin A storage.[2]

Comment

The protein content of liver can be decreased by diets low in protein (26, 27), which may therefore interfere with the hepatic storage of vitamin A by lowering the liver component to which vitamin A is attached. The concept of a vitamin A protein has already been suggested in connection with the retina, where vitamin A apparently exists as a modified component of visual purple (28). Cytologists point out a close association of vitamin A with the nucleolus (29) and with the mitochondria (30–32). Since vitamin A storage does not appear to be directly associated with carcinogenesis (3, 33), it is suggested that the observed variations in hepatic vitamin A, whether due to hydrocarbon, alcohol, or low protein, are indicative of a basic chemical alteration in some liver constituent other than the vitamin itself. It is even possible that vitamin A may become useful in the detection of intracellular changes too delicate for measurement by other means.

The rôle of the kidney in vitamin A metabolism is probably more important than has hitherto been supposed. In vitamin A deficiency in dogs urea clearance is well below normal (34). Vitamin A has been identified in the urine of dogs exposed to anesthetics (35) and in certain pathological human urines (36), particularly those involving renal and hepatic dysfunction (37). The hepatic

[2] Protocols of the experiments on the extent of the vitamin A shift to the kidney, on the effect of vitamin E, and on certain additional experiments with diets low in protein will be furnished on request.

storage of vitamin A is markedly subnormal in patients suffering from chronic nephritis (38, 39) and the levels of the vitamin in the blood are relatively high in these individuals (40, 41). During a fast, the proteins of the kidney are retained to a much greater degree than the proteins of the liver (42). This parallels the observation that in the presence of dibenzanthracene the ability to hold vitamin A is retained to a much greater degree by the kidney than by the liver.

SUMMARY

1. The injection of dibenzanthracene reduced the amount of vitamin A in the livers of rats and increased the amount in the kidneys. Methylcholanthrene promoted a similar shift of vitamin A. In animals injected with dibenzanthracene as much as 50 per cent of the total body vitamin A was found in the kidneys, although the percentage never exceeded 5 per cent in uninjected animals. However, the increased vitamin in the kidney accounted for only about 10 per cent of that which had left the liver.

2. The ingestion of 15 per cent alcohol, like the injection of dibenzanthracene, increased the rate of depletion of vitamin A from the liver, but to a lesser extent than did the hydrocarbon. Alcohol did not promote an increase in the vitamin content of the kidney.

3. The effect of the dibenzanthracene was not counteracted by cystine, nor by diets high or low in vitamin E. Bromobenzene and anthracene fed at low levels failed to alter vitamin A storage.

4. Diets low in protein reduced the storage of vitamin A in the liver and increased the rate at which stored vitamin A was depleted. The results are in harmony with the suggestion that the vitamin is held in the liver in the form of a protein complex.

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THE LABILITY TOWARD ALKALI OF SERINE AND THREONINE IN PROTEINS, AND SOME OF ITS CONSEQUENCES

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(Received for publication, October 24, 1941)

Some years ago, one of us (1) discussed in some detail the probable mechanism of the reaction by which cystine is split by alkali, and the reasons why this splitting occurred so much more readily in peptide combination.

The somewhat hypothetical system there considered suggests the possibility that serine and threonine may also show (in protein combination) a similar lability, probably *equally enhanced* from the greater inactivity which these acids show in the free state, but surely less than that of analogous cystine derivatives. With the aid of analytical methods which we have developed for serine (2) and threonine (3) it has become possible to test such a prediction, and we here report the results of this test (4).

We find that serine and threonine are indced subject to partial destruction when in protein combination, *under conditions which do not measurably affect them in the free state*, and that this destruction takes the form of the elimination of water, to form (in the case of serine) what is presumably a dehydroalanyl grouping in the protein. In a subsequent paper we shall complete the proof by showing that the hypothesized dehydroalanyl derivative can add sulfur compounds to form products which are demonstrably cystine derivatives.

Standardization of Whole Silk—It was first necessary to establish the serine and threonine content of the whole silk used as a reference. We were sufficiently fortunate to obtain a sample of *Bombyx mori* "hard" silk closely similar to that from which the sericin and fibroin which we analyzed (5) were prepared, together with the

information that it contained 18.5 per cent of sericin. It had been thoroughly extracted with alcohol and ether. This was dried for 1 hour at 105° in a vacuum oven before use.

There was a possibility that the analyses previously reported (5) for sericin might represent a mixture of substances which did not in themselves accurately represent the original sericin of the silk. But the sericin in the fat-free whole silk can scarcely have undergone much alteration. Table I shows the results obtained on the whole silk, compared with those (assuming 18.5 per cent sericin) calculated from our previously reported (5) results. The agreement is sufficiently good to offer substantial confirmation of our earlier values.[1]

Table I

Comparison of Serine and Threonine in Whole Silk with Results Previously Reported (5) for Fibroin and Sericin

The results are expressed in milliequivalents per gm. This unit is preferable to "serine equivalents" used in previous papers.

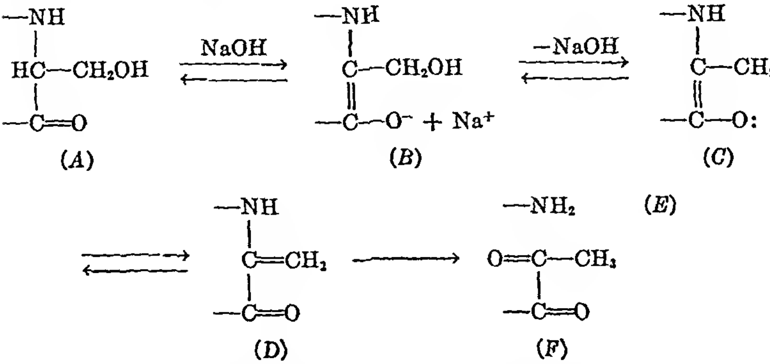
	Fibroin found	Sericin found	Whole silk	
			Found	Calculated
Threonine	0.129	0.85	0.236	0.262
Serine	1.291	3.22	1.556	1.650
Total hydroxyamino acids	1.414	4.17	1.935	1.926

Lability of Standard Silk—In all the experiments described, the protein was refluxed for 1 hour in an atmosphere of nitrogen with 0.1 N sodium hydroxide, and subsequently hydrolyzed for 20 hours with constant boiling hydrochloric acid. In Run A in Table II, the *volume* of alkali was insufficient, but the results are worth recording. In the Runs B and C, the volume of alkali was larger, and the ratio of alkali volume to weight of silk was such as to make these essentially duplicates, except for the time of heating.

Having established the expected lability at least qualitatively, it occurred to us that there was a necessary consequence. If it were true that the reactions discussed yield eventually peptides of dehydroamino acids, it should follow that these, on acid hy-

[1] We wish to thank Dr. Milton Harris, of the Textile Foundation, National Bureau of Standards, for the silk, the sericin determination, and the suggestion.

drolysis, should yield ammonia, and that this should appear as an addition to the "amide ammonia." The data in Table III indicate that this is the case.



It will be seen that the additional ammonia liberated on acid hydrolysis after treatment with alkali accounts for more than 91

TABLE II

Effect of Alkali on Hydroxyamino Acids of Whole Silk

The results are expressed in milliequivalents per gm.

Run	Total hydroxyamino acids	Threonine	Serine
Original	1.94	0.236	1.556
A	1.56 (20)	0.164 (30)	1.340 (14)
B	1.26 (35)	0.120 (49)	1.116 (28)
C	1.25 (35)	0.124 (47)	1.048 (33)

Run A, 2.37 gm. of silk with 34 cc. of 0.1 N NaOH. Run B, 2.00 gm. of silk with 60 cc. of 0.1 N NaOH; refluxed 2 hours. Run C, same as Run B, but refluxed 1 hour.

The figures in parentheses represent the percentage decrease from the original values.

per cent of the loss of serine and threonine. We are inclined to interpret these results as an approximate proof for the dehydro structure of the intermediate products in the indicated reaction.

Assuming the reaction of (for instance) serine in peptide form to resemble that of cystine, it may be formulated as indicated in the equations shown. Some suitable region of the protein mole-

cule A undergoes, in the alkaline solution, enolization to B. We should reach the same result whether we spoke of vinylogy or of 1,4-elimination. The fact remains that B contains an anion which can discharge itself by the loss of hydroxyl ion from the β-carbon atom. The product C can stabilize itself, relatively, as D, but numerous resonance possibilities supervene, and the reactions are all more or less readily reversible.

D eventually probably splits to give an acid amide, E, which will contribute additional ammonia to the "amide ammonia" when hydrolyzed, and a pyruvyl derivative of some peptide fragment. We have at present no way of knowing *much* about the stability of D. We do, however, have reason to know that it should at least be considerably more stable than its simplest relative, aminoacrylic acid. And its persistence, at least for a moderate length of time, is strongly indicated by the addition reactions to be reported elsewhere.

TABLE III

Balance Sheet for Hydrolyzable Ammonia in Whole Silk

The results are expressed in milliequivalents per gm.

	Threonine	Serine	Ammonia
Original	0.236	1.556	0.511
Treated	0.124	1.048	1.077
Change	−0.112	−0.508	0.566*
	0.566 − 0.620 = −0.054		

* Additional ammonia liberated on acid hydrolysis.

The end-products of the series of reactions given are entirely consistent with the demonstrated reactions of cystine and of serine, when these are not combined. The enormously increased rate of *both* of these reactions, as compared to those of the free amino acids with alkali, strongly implies a kindred mode of activation. The fact that the expected "additional ammonia" is nearly quantitatively developed seems to us a confirmation of the course suggested for these reactions, and also for our previous speculations (1) on cystine derivatives, for it is a direct consequence of the interpretation we have developed on quite other grounds, and it does not appear to be called for by most other explanations.

SUMMARY

1. We have demonstrated a reaction by which serine and threonine, when in protein combination, are destroyed in alkaline solution at a rate which is disproportionately greater than any corresponding effect on free serine or threonine.

2. This reaction has been interpreted in some detail, along the lines previously discussed for cystine.

3. The liberation of "additional ammonia," beyond that usually described as "amide ammonia" in the *direct* hydrolysis, has been shown to be quantitatively correlated with the destruction of hydroxyamino acids.

4. We believe that the reaction in question involves (*e.g.*, for serine) the formation of dehydroalanyl units in the not too greatly decomposed protein molecule.

5. We expect to offer evidence in a subsequent paper that *only* such an assumption would explain the results obtained.

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THE ISOLATION OF BIOTIN FROM MILK

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With the isolation of biotin from a vitamin H liver concentrate (1) other sources were investigated for the preparation of biotin on a larger scale. One of the most suitable starting materials was found to be a biotin concentrate prepared by the S. M. A. Corporation from milk.[1] In this paper we wish to describe the procedure which was found satisfactory for the isolation of pure biotin from this material.

The milk concentrates which we obtained contained 1 to 2 mg. of biotin per gm. of solids. For further purification the biotin concentrates were treated with acidic methanol and the biotin methyl ester so obtained was subjected to chromatographic adsorption procedures, first Decalso and then activated alumina being used as the adsorbent. The crude crystalline biotin methyl ester which was obtained from the eluates of the activated alumina was purified by washing with ethyl acetate, by sublimation *in vacuo*, and by crystallization from a mixture of methanol and ether. Pure crystalline biotin was obtained from the methyl ester by saponification with dilute alkali and acidification of the saponification mixture with HCl.

Based on the biotin concentrates used as the source material, a 500- to 1000-fold purification of biotin was accomplished with a yield of over 25 per cent. This yield was raised to 35 or 40 per

[1] We wish to thank Mr. W. O. Frohring of the S. M. A. Corporation, who first suggested this material as a source of biotin and for a research grant which has aided greatly in this work. We also wish to express our appreciation to the Research Laboratories of the S. M. A. Corporation for placing at our disposal large quantities of biotin concentrates.

cent by purification of the various side fractions obtained from the esterification and adsorption steps.

EXPERIMENTAL

The biotin contents of the various fractions obtained during the isolation procedure were determined by the yeast growth method (1, 2), with pure biotin as a standard.

Esterification of Crude Biotin Concentrate—An aqueous solution of 105 gm. of biotin concentrate which contained 85 mg. of biotin (0.08 per cent purity) was concentrated to a syrup *in vacuo* and the residue was dissolved in 250 cc. of methanol. The insoluble material was removed by filtration and the filtrate was concentrated to a thick syrup *in vacuo*. The concentration with methanol was repeated to remove the last traces of water. The residue was dissolved in 500 cc. of absolute methanol and 40 gm. of dry HCl gas were passed into the solution which was then refluxed gently for 1 hour. The esterification mixture was concentrated to a thin syrup *in vacuo*, and to the residue were added 200 cc. of water and enough solid $NaHCO_3$, with shaking, to make the solution alkaline to litmus. The alkaline solution was extracted five times with 200 cc. portions of ethyl acetate. The combined ethyl acetate extracts were dried over Na_2SO_4, filtered, and concentrated *in vacuo* to remove the ethyl acetate. The residue weighed 45 gm. and contained 67 mg. of biotin (0.15 per cent purity). The yield of biotin methyl ester in this step was 79 per cent. This was raised to approximately 90 per cent by reesterification and extraction of the water residue.

Decalso Adsorption of Biotin Methyl Ester—55 gm. of material from the esterification procedure, containing 81 mg. of biotin methyl ester, were dissolved in 400 cc. of chloroform and this solution was passed through an adsorption column packed with 250 gm. of 60 to 100 mesh Decalso in chloroform. The column was then washed in succession with 3600 cc. of chloroform and 1200 cc. of a solution of 5 per cent methanol in acetone. The methanol-acetone eluate, which contained 66 mg. of biotin methyl ester and 10.7 gm. of solids (0.62 per cent biotin), was concentrated *in vacuo* to remove the solvents and the residue was dissolved in 500 cc. of acetone for purification in the next step.

Alumina Adsorption of Biotin Methyl Ester—The acetone solu-

tion of the biotin ester was passed through a column prepared from 220 gm. of activated alumina (Alorco Grade A, 120 to 150 mesh) in acetone. The column was then washed in succession with 2300 cc. of acetone, 900 cc. of a solution of 10 per cent methanol in acetone, 350 cc. of methanol, and 400 cc. of a solution of 20 per cent concentrated NH_4OH in methanol. The first 500 cc. of the methanol-acetone eluate were collected in separate 50 cc. portions. The distribution of biotin methyl ester in the various fractions is shown in Table I.

TABLE I

Distribution of Biotin Methyl Ester in Alumina Chromatograph

Fraction No.	Solvent		Weight	Biotin	
		cc	*mg*	*mg.*	*per cent*
634-I	Acetone	2300	3000	0.03	0.001
634-II	Methanol-acetone	50	327	0.06	0.02
634-III	"	50	227	6.30	2.8
634-IV	"	50	140	14.80	10.5
634-V	"	50	81	15.10	18.6
634-VI	"	50	65	10.00	15 4
634-VII	"	50	52	6.35	12.2
634-VIII	"	50	53	3.50	6.6
634-IX	"	50	48	2.00	4.2
634-X	"	50	43	0.50	1.2
634-XI	"	50	41	0.30	0 70
634-XII	"	400	363	2.05	0.56
634-XIII	Methanol	350	1210	1.33	0.11
634-XIV	NH_4OH-methanol	400	3500	4 15	0.12
Total...... ..			.	66.47	

Each of the methanol-acetone fractions was concentrated *in vacuo* to remove the solvent. Crystals of biotin methyl ester appeared in Fractions 634-V and 634-VI when 1 or 2 drops of methanol were added to the residues. Biotin methyl ester crystallized from Fractions 634-IV and 634-VII when the residues were seeded with crystalline ester. Fractions containing less than 10 per cent biotin did not crystallize. The semicrystalline residues were allowed to stand for several hours, during which time more biotin methyl ester crystallized.

Purification of Crystalline Chromatograph Fractions—Semi-

crystalline residues containing a total of 118 mg. of biotin methyl ester were washed with 1 cc. portions of ethyl acetate until the washings were colorless. Almost all the colored material which was present was removed by this procedure. The insoluble crystalline biotin methyl ester, 82 mg. melting at 163–164°, was further purified by sublimation at 150–160° and 10^{-5} mm. pressure. The sublimate, weighing 81 mg., was dissolved in the minimum amount of hot methanol and 10 volumes of peroxide-free ether were added in portions to the solution. Biotin methyl ester separated from the solution in long thin needles. The solvent was removed and the crystals were washed with ether-methanol and then with ether. The yield of pure biotin methyl ester was 70 mg., micro melting point 166–167° (uncorrected). Additional amounts of the ester were obtained by concentration of the mother liquors.

Preparation of Free Biotin—Free biotin was prepared from the ester as previously described (3). 85 mg. of pure biotin methyl ester were dissolved in 5 cc. of 1 N NaOH by warming. The clear solution was concentrated *in vacuo* to approximately 2 cc. and made acid to Congo red with dilute HCl. Free biotin crystallized from the solution. The crystals were washed with cold water and dried *in vacuo*. The yield of free biotin, micro melting point 230–232° (uncorrected), was 80 mg.

The authors wish to express their appreciation to Mr. Joseph Karabinos and Mr. Karl Dittmer of this laboratory for aid in carrying out many of the isolation steps.

SUMMARY

A method for the isolation of pure crystalline biotin from a milk concentrate has been described. The yield of biotin is 25 to 40 per cent. The method is suitable for the preparation of relatively large amounts of pure biotin.

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THE FRACTIONATION OF CATTLE BLOOD IODINE WITH ALCOHOL

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(Received for publication, August 8, 1941)

Blood iodine has been frequently separated into alcohol-soluble and alcohol-insoluble fractions. There is lack of agreement concerning the physiological significance of these fractions. There is even lack of agreement concerning how much blood iodine is alcohol-soluble and how much is alcohol-insoluble. Some have found that practically no blood iodine is soluble in alcohol, others that practically all blood iodine is soluble in alcohol, while the majority of investigators have reported an intermediate distribution. This chaotic state of affairs has been amply discussed in recent monographs such as those of Elmer (1) and Salter (3). In the present investigation, two factors were found to affect the solubility of blood iodine in alcohol, namely heat and hydrolysis, and it is suggested that variation in the degree of influence of these factors may account for at least part of the discrepancies of published reports.

Oxalated blood of eight cattle was obtained from the slaughterhouse and its total iodine estimated by Perkin's (2) method, which had been previously found to yield a 90 per cent recovery of iodine added in amounts of from 0.13 to 1.3 γ, with decreasing recovery of lesser amounts; 0.03 γ was the smallest amount which could be detected. The average total blood iodine on duplicate analyses of each sample was 4.16 $\pm$ 1.6 (mean $\pm$ standard deviation) γ per 100 ml. of whole blood (Table I). Quadruplicate samples of 25 ml. of blood from each of the eight cattle were repeatedly extracted with alcohol at room temperature until two successive extracts were found iodine-free. The total amount of iodine dissolved by this "cold" alcoholic extraction (Extract A) averaged

1.88 ± 0.44 γ per cent and the average amount left in the residues (Residue A) was 2.04 ± 0.64 γ per cent. These figures correspond to those of the majority of previous reports.

Another set of duplicate Residues A was prepared in a similar manner and at the same time as the above. It was extracted with hot alcohol in a Soxhlet apparatus until two successive 24 hour extracts were iodine-free. The total amount of iodine extracted with this hot alcohol (Extract B) was 1.36 ± 0.56 γ per cent and the mean iodine content of the Soxhlet residues (Residue B) was 0.64 ± 0.40 γ per cent. The two methods of extraction, "cold" and hot alcohol, removed a mean of 78 per cent of the original total blood iodine, which again corresponds to the values reported

TABLE I

Alcoholic Extraction of Iodine from Cattle Blood

Material analyzed	Fractionation procedure	Fraction	Iodine content (mean ± standard deviation)	Per cent of total iodine
			γ per 100 ml. whole blood	
Whole blood	None	None	4.16 ± 1.6	100
" "	Cold alcohol	Extract A	1.88 ± 0.44	45
" "	" "	Residue "	2.04 ± 0.64	49
Residue A	Hot "	Extract B	1.36 ± 0.56	33
" "	" "	Residue "	0.64 ± 0.40	15
" "	Dry heat + cold alcohol	Extract C	0.88 ± 0.64	21
" "	" " + " "	Residue "	1.52 ± 1.12	37

by most previous investigators, but it is upon this percentage that there is the most lack of agreement, some finding that all blood iodine can be so extracted.

Those who have shown that hot alcoholic extraction removes more iodine from blood than cold alcoholic extraction usually explain that this is due to a greater penetrating and solvent power of the hot alcohol. It seemed possible to us that heat itself might alter some of the cold alcohol-insoluble compounds of iodine in such a way as to make them soluble in alcohol. There might be two fractions of blood iodine, one immediately soluble in cold alcohol and another made soluble in cold alcohol after the application of dry heat. Since heat affects proteins, it might alter an

iodine fraction or fractions associated with proteins, either physically or chemically.

Quadruplicate Residues A of each of the eight samples of cattle blood were heated in an oven at 78°. At the end of each succeeding period of 24 hours, the residues were removed from the oven, cooled to room temperature, triturated, and extracted with alcohol at room temperature until no further iodine was extracted. At the end of the 4th day, the iodine content of the residues (Residues C) was estimated.

Dry heat made soluble in cold alcohol (Extract C) some of the iodine of Residue A which was originally insoluble in cold alcohol. Most of the iodine of Extract C dissolved after 1 day's heating (average 59 per cent), lesser amounts after 2 days (average 27 per cent) and 3 days (average 14 per cent), and no more on the 4th day. The average total amount of iodine of Extract C was $0.88 \pm 0.64\ \gamma$ per cent. Owing to the large variation consequent upon the small amounts of iodine being determined and the summation of manipulative errors, the quantitative significance of these figures is not great but the results do indicate that heat itself is a factor which affects the solubility of blood iodine in alcohol and which probably affects the amount of iodine extracted with hot alcohol in a Soxhlet apparatus. The iodine content of Residue C was $1.52 \pm 1.12\ \gamma$ per cent.

Quadruplicate Residues C were prepared from 25 ml. samples of dog blood and subjected to an additional 48 hours heating at 120° but no more iodine could be made soluble in cold alcohol and the final residues contained no less iodine than those heated at 78° for 4 days.

Quadruplicate Residues B were then prepared in a similar manner from each of two new samples of cattle blood. Their average iodine content was $0.97\ \gamma$ per cent. Half of them was hydrolyzed by heating with 100 ml. of 95 per cent alcohol and 5 ml. of a saturated solution of sodium hydroxide in distilled water on a steam bath until dry. They were then cooled, triturated, and extracted with cold alcohol. No detectable iodine was found in the residue after hydrolysis and all of the original iodine of whole blood could be accounted for, within the limits of error of the method, in the various alcoholic extracts. In no residue was any iodine found after alkaline, alcoholic hydrolysis. These

results indicate that hydrolysis is also a factor which affects the solubility of blood iodine in alcohol and which also might be responsible for varying degrees of extraction of iodine in a Soxhlet apparatus.

SUMMARY

It was found that that fraction of blood iodine which is insoluble in cold alcohol can be made partially soluble in cold alcohol (*i.e.* alcohol at room temperature) by the application of dry heat to the residue and completely soluble in cold alcohol after the application of heat and alcoholic sodium hydroxide to the residue. It is suggested that varying degrees of influence of heat and hydrolysis may account at least in part for the discrepancies in reported values of hot alcohol-soluble iodine of blood.

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STUDIES ON THE URINARY EXCRETION OF PANTOTHENIC ACID

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In connection with studies on the toxicology and pharmacodynamic action of pantothenic acid (1), its absorption and excretion were investigated by a microbiological assay with *Lactobacillus casei* as the test organism. Simultaneous determinations of riboflavin were also made, again with *Lactobacillus casei*. The experiments were performed on dogs with bladder fistula which received crystalline dextrorotatory calcium pantothenate, either orally or intravenously. Blood and urine samples were taken at frequent intervals for the determination of both pantothenic acid and riboflavin, and each experiment was performed in duplicate.

The dogs were maintained on a diet of Purina dog chow. After overnight fasting, the dogs received 200 ml. of water by stomach tube 3 hours before the experiment. Simultaneously with the administration of the vitamin a second dose of 250 ml. of water was given orally in order to obtain sufficient quantities of urine at frequent intervals. Blood samples were taken from the veins of the front or hind legs at 5 to 20 minute intervals throughout the 2 hour duration of the experiments. The urine samples were collected in dark bottles under a layer of toluene and kept in the refrigerator; all samples were acid (pH 6.0 to 6.7). Whole blood samples were laked (1 ml. in 19 ml. of water) and preserved in the same manner.

Assay Method

The method used for the pantothenic acid assays was essentially that of Snell and Strong for riboflavin (2) with the exception that the yeast extract supplement was replaced with thiamine (10 γ per tube), pyridoxine (10 γ), nicotinic acid (10 γ), and riboflavin

(1 γ). Assays with this medium agreed within the experimental error with similar assays in which yeast extract supplement was used.

In order to minimize any inhibiting effects of urine and laked blood, the samples were assayed in portions as small as possible. Less than 1 ml. of undiluted dog urine per tube gave satisfactory results with or without added calcium pantothenate. However, with blood the assays were inconsistent even when less than 0.1 ml. of whole blood was used. Therefore the normal blood figures do not represent absolute values; those after dosing are more nearly absolute, since the dilutions necessary for assay of these bloods minimize the effects of interfering substances. In the assay of normal blood it was found necessary to add 0.025 to 0.075 γ of calcium pantothenate to each tube in order to make possible readings on the reliable portion of the standard curve. The mass of blood proteins which precipitated during autoclaving was broken into small pieces by vigorous shaking before inoculation and twice every 24 hours thereafter. After 40 to 72 hours incubation the tubes were simultaneously cooled in an ice water bath before titration with 0.1 N alkali; phenolphthalein was used as indicator. Stanbery *et al.* (3) used a similar procedure for the assay of human blood.

Riboflavin assays were performed exactly as were those for pantothenic acid except that the riboflavin supplement of 1 γ per tube was replaced by 1 γ of calcium pantothenate.

EXPERIMENTAL

Table I presents data on the excretion of pantothenic acid and riboflavin in control experiments in which only water was given. The onset of diuresis had a diluting effect on the content of both vitamins in the urine, but the amount of each vitamin excreted per unit time remained fairly constant. The total amount of pantothenic acid excreted in four such experiments averaged 7.5 γ for the 2 hours. The actual figures were 7.3, 8.3, 7.7, and 6.6 γ of pantothenic acid. For riboflavin the figures were 11.9, 10.0, 3.8, and 11.0 γ with an average of 9.2 γ in 2 hours.

Data on the excretion of pantothenic acid after oral and intravenous administration of 1 or 4 mg. per kilo of body weight respectively are given in Table II. No significant increase in the

TABLE I

Pantothenic Acid and Riboflavin in Urine

The dogs (Nos. 101 and 90) received 250 ml. of water at 0 minute.

Time	Volume per 20 min.		Pantothenic acid				Riboflavin			
	Dog 101	Dog 90	Dog 101	Dog 90	Dog 101	Dog 90	Dog 101	Dog 90	Dog 101	Dog 90
min.	*ml.*	*ml.*	*γ per ml.*	*γ per ml.*	*γ per 20 min.*	*γ per 20 min.*	*γ per ml.*	*γ per ml.*	*γ per 20 min.*	*γ per 20 min.*
−20– 0	15.0	16.0	0.13	0.4	2.0	6.4	0.09	0.03	1.4	0.5
0– 20	16.5	4.5	0.07	0.24	1.2	1.1	0.12	0.17	2.0	0.8
20– 40	26.0	18.5	0.05	0.08	1.3	1.5	0.07	0.12	1.8	2.2
40– 60	47.5	17.5	0.05	0.09	2.4	1.6	0.05	0.10	2.4	1.8
60– 80	38.5	32.0	0.015	0.04	0.6	1.3	0.05	0.09	1.9	2.9
80–100	35.0	16.5	0.04	0.03	1.4	0.5	0.06	0.08	2.1	1.3
100–120	17.0	29.0	0.025	0.02	0.4	0.6	0.1	0.07	1.7	2.0
Excreted in 2 hrs., γ					7.3	6.6			11.9	11.0
" " 2 " (duplicate experiments), γ					8.3	7.7			10.0	3.8

TABLE II

Urinary Excretion of Pantothenic Acid per 20 Minutes Following Oral and Intravenous Administration of 1 Mg. and 4 Mg. of Calcium Pantothenate Respectively per Kilo

The dogs (No. 101, 14 kilos and No. 90, 10 kilos) received 250 ml. of water orally at 0 minute.

Time	Dog 101					Dog 90	Dog 101	Dog 90
	1 mg. orally		4 mg. orally		1 mg. intraveoously		4 mg. intraveoously	
min.	γ	γ	γ	γ	γ	γ	γ	γ
−20– 0		0.4	0.7	0.4	1.4	0.3	2.3	2.1
0– 20	3.9	0.6	2.0	0.6	3710.0	1050.0	16,000.0	6640.0
20– 40	3.1	1.1	4.6	15.2	640.0	1140.0	12,010.0	5380.0
40– 60	7.6	1.3	17.0	380.0	43.0	10.4	2,920.0	2930.0
60– 80	3.5	1.7	240.0	780.0	9.3	1.7	960.0	700.0
80–100	6.1	2.3	150.0	870.0	3.9	1.9	200.0	190.0
100–120	5.3	2.0	95.0	720.0	2.5	1.6	50.0	370.0
Excreted in 2 hrs., *mg*	0.03	0.009	0.5	2.8	4.4	2.2	32.1	16.2
% of dose excreted in 2 hrs.	Negligible		0.9	5.0	31.4	22.0	57.3	40.5
Riboflavin excreted in 2 hrs., γ		6.8	6.3	6.7	5.9	4.4	6.6	3.6

excretion of the vitamin was found after oral administration of 1 mg. per kilo and no rise in the blood level was observed. However, after 4 mg. per kilo orally there was a transient rise in the blood level 20 minutes after ingestion of the vitamin. 10 minutes later the blood content returned to its previous level, and the urinary excretion increased gradually to a maximum during the 60 to 100 minute period. 1 to 5 per cent of the orally administered vitamin was found in the urine.

TABLE III

Pantothenic Acid and Riboflavin in Blood and Urine after Intravenous Administration of 4 Mg. per Kilo of Calcium Pantothenate or Sodium Riboflavinate

Dog 101, female, 14 kilos, received 250 ml. of water orally at 0 minute.

Time	Calcium pantothenate				Sodium riboflavinate			
	Pantothenic acid		Riboflavin		Pantothenic acid		Riboflavin	
	Urine	Blood	Urine	Blood	Urine	Blood	Urine	Blood
min.	*γ per 20 min.*	*γ per ml.*	*γ per 20 min.*	*γ per ml.*	*γ per 20 min.*	*γ per ml.*	*γ per 20 min.*	*γ per ml.*
0	2.3	2.2	2.4	1.1	3.1	1.0	0.9	1.2
5		26.0		0.9		1.1		11.0
10		16.0		0.9		1.0		6.0
20	16,000.0	9.0	1.1	1.0		0.9	2000.0	5.4
40	12,010.0	4.4	0.9	1.0	2.2	1.0	8610.0	2.5
60	2,920.0	3.4	1.3	1.0	1.6	0.9	6000.0	2.1
80	960.0	3.1	1.5	1.1	1.4	0.8	3540.0	1.7
100	200.0	2.6	0.9	1.1	1.0	1.2	950.0	1.7
120	50.0	2.3	0.90	1.1	1.0		680.0	1.6
Excreted in 2 hrs., *mg.*	32.1		0.007		0.007		21.8	

After intravenous injection the amount of the vitamin in the urine was markedly higher. After 1 mg. per kilo intravenously 22 to 31 per cent was excreted and after 4 mg. per kilo 41 to 57 per cent. Since in all four experiments the peak in the excretion was passed before the end of the 2 hour period, the totals represent fairly close approximations of the fraction of each dose excreted.

No significant influence of the administration of calcium pantothenate on either the blood level or urinary excretion of riboflavin

was revealed in these experiments. The 2 hour excretion of riboflavin (bottom, Table II) remained essentially the same as in the controls (Table I).

In Table III reciprocal experiments with intravenous administration of 4 mg. of calcium pantothenate per kilo and 4 mg. of sodium riboflavinate respectively are compared. In both experiments a striking increase in the blood level of the injected vitamin was induced with gradual return to approximately normal levels within 2 hours. A large fraction of the administered vitamin was excreted in each case (riboflavin 39 per cent, pantothenic acid 57 per cent). In duplicate experiments with Dog 90 similar results were obtained with 48 per cent of the riboflavin and 41 per cent of the pantothenic acid found in the urine. The administration of either vitamin did not significantly affect the blood levels or urinary excretion of the other.

DISCUSSION

Axelrod, Spies, and Elvehjem (4), in a study of human riboflavin excretion, administered 200 γ per kilo of body weight intravenously to three subjects and found 30 to 40 per cent in the urine in the 1st hour. This is in agreement with our results after a dose of 4 mg. of riboflavin per kilo of body weight in dogs. With pantothenic acid, comparable excretion was observed after the intravenous administration of 1 or 4 mg. of calcium pantothenate per kilo.

After oral administration of calcium pantothenate (1 or 4 mg. per kilo, Table II) 0 to 5 per cent was excreted in the urine and when the same doses were given intravenously, 22 to 31 per cent of the smaller dose and 41 to 57 per cent of the larger were excreted. These results are in direct contrast with results obtained in a similar investigation of the excretion of pyridoxine by the dog (5). After doses of 25 to 500 mg. of pyridoxine per dog, about 20 per cent of the dose was consistently found in the urine regardless of the method of administration.

Spies and coworkers (6) have reported that intravenous injections of 100 mg. of pantothenic acid cause a temporary rise of 20 to 30 per cent in the riboflavin concentration in the blood of normal persons and that similarly the injection of riboflavin produces a rise of 45 per cent in the pantothenic acid level of the blood. In

our experiment on dogs we did not obtain evidence for such an interaction of riboflavin and pantothenic acid.

SUMMARY

1. The 2 hour excretion of riboflavin and pantothenic acid during diuresis in two dogs with bladder fistula averaged 7.5 γ of pantothenic acid and 9.2 γ of riboflavin.

2. After an oral dose of 1 mg. of calcium pantothenate per kilo of body weight no significant fraction of the dose was excreted in 2 hours. When the dose was increased to 4 mg. per kilo, however, a distinct rise in the urinary pantothenic acid was observed with a peak at 60 to 100 minutes and with a 2 hour excretion of 0.9 to 5.0 per cent.

3. When 1 mg. of calcium pantothenate per kilo was injected intravenously, 22 to 31 per cent of the dose was found in the urine with a peak in the excretion within 40 minutes. After 4 mg. per kilo intravenously 41 to 57 per cent was excreted in the urine with a peak within 20 minutes.

4. The blood level of pantothenic acid rose markedly after the intravenous administration of 4 mg. per kilo but rapidly returned to normal in 2 hours.

5. No effect of the administration of calcium pantothenate on the blood or urine levels of riboflavin was observed. Similarly, after administration of 4 mg. of sodium riboflavinate per kilo intravenously, the pantothenic acid content of the blood and urine showed no significant changes.

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BIOLOGICAL SYNTHESIS OF CHOLINE BY RATS ON DIETS WITH AND WITHOUT ADEQUATE LIPOTROPIC METHYL*

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The curious fact that the choline content of the livers of rats is essentially constant, irrespective of whether this substance is supplied in the diet or not, or whether fatty liver is present or not, has been noted by several observers (1–3). To explain this anomaly it is necessary to suppose that in animals on choline-low diets either the catabolism of choline is restricted or choline is synthesized by the animal in a quantity sufficient to maintain its normal content. An indication of the extent of choline synthesis in growing rats on choline-free diets has recently been given by Jacobi, Baumann, and Meek (3).

The fragments from which the rat synthesizes choline are now all identified. Du Vigneaud *et al.* (4) have shown that the methyl group of methionine is transferred intact, and the present writer (5) has shown that ethanolamine is the substrate that is methylated. An insufficiency of ethanolamine appears unlikely in view of the demonstrated ability of animals to make this compound by the reduction of glycine (5) or possibly by the decarboxylation of serine (6). As du Vigneaud and his collaborators have proved that it is indeed deficiency of available methyl groups in the diet that is involved in the syndrome of fatty liver (4), it was decided to investigate the effect of diets rich and poor in methyl groups upon the quantity of choline that rats could prepare by the methylation of isotopically labeled ethanolamine.

To this end two groups of three adult male rats were placed on a

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low protein, high carbohydrate diet, poor in methionine. To Group I additional guanidoacetic acid and normal ethanolamine hydrochloride were fed in the hope of depleting the body reserves of available methyl groups (7, 8, 4, 5). To Group II betaine hydrochloride was fed to serve as a source of available methyl groups (5, 9, 10). After a week ethanolamine hydrochloride containing

TABLE I

Ratios of Isotope Content

Three adult male rats, in each experiment, on the same low protein basal diet, were given the dietary supplements in the quantities and for the periods indicated. The isotope content of the various substances isolated was computed on the basis of 100 atom per cent in the isotopic ethanolamine hydrochloride fed.

Dietary supplement		Group I	Group II
		mM per rat per day	*mM per rat per day*
Preliminary 7 days	Ethanolamine HCl	1.5	
	Guanidoacetic acid	0.2	
	Betaine HCl		1.5
Final 3 days	Ethanolamine HCl, N^{15}	1.5	1.5
	Guanidoacetic acid	0.2	
	Betaine HCl		1.5
Compounds isolated		**N^{15} content**	
		atom per cent	*atom per cent*
From total phosphatides	Choline (*A*)	5.8	8.7
	Ethanolamine (*B*)	9.3	16.4
From organ proteins	Glutamic acid	1.2	1.3
	Glycine	0.6	0.5
Ratio *A*:*B*		0.62	0.53

N^{15} was added to both diets, the *normal* substance being withdrawn from the diet of Group I.

After 3 days the animals were killed and ethanolamine as well as choline isolated from the body phosphatides. Glycine and glutamic acid were isolated from the proteins of the internal organs. The N^{15} determinations on these compounds are reported in Table I, the same conventions being employed as in the previous report (5).

As had been intended, the animals in Group I had grossly fatty livers at the time of death, whereas those in Group II, being amply supplied with betaine, had no apparent abnormalities.

The ethanolamine isolated in Group I is appreciably lower in isotope than that isolated in Group II. The difference may be ascribed to the fact that during the preliminary feeding the animals in Group I were receiving normal ethanolamine, in contrast to those in Group II. Such an explanation suggests that when ethanolamine is present in the diet in moderate quantity, the animal may be able to store it, possibly with an increase in the content of cephalin. This would result in a dilution of the isotopic ethanolamine that was fed later, and a correspondingly lower analytical figure.

The ratios of isotope in choline to that in ethanolamine isolated differ but little in the two series. If it be assumed that the choline was formed by the methylation of ethanolamine of the same isotopic composition as that in the phosphatide, it follows that in both cases about half (62 and 53 per cent) of all the choline in the animal had been thus synthesized in 3 days. There was no significant difference in spite of the fact that the first diet was deficient in available methyl groups and resulted in fatty liver, while the second diet was adequately supplied with methyl groups. A similar result had also previously been obtained with rats on a high protein diet (5).

It therefore appears that even in methyl group deficiency severe enough to produce fatty liver, the methylation of ethanolamine to produce choline proceeds unimpeded in the body of the rat. That this is not simply the reversible shifting of methyl groups from one ethanolamine residue to another is shown by the fact that choline, when fed, is but slowly if at all demethylated to ethanolamine (5). There is no doubt that choline may serve as a biological source of methyl groups (4), but apparently ethanolamine is not the resulting product.

These results, in confirmation of those of Jacobi, Baumann, and Meek (3), serve to explain the independence of the choline content of the body from the amount of choline in the diet, referred to previously (1–3). Presumably the quantity of choline in the body is maintained at a constant level, when this material is lacking in the diet, by the biological methylation of ethanol-

amine. This reaction proceeds even when there is a degree of methyl group deprivation sufficient to produce fatty liver.

EXPERIMENTAL

The basal diet comprised 5 parts of arachin[1] (11), 2 parts of yeast powder, 4 parts of salt mixture (12), 6 parts of Wesson oil, 2 parts of cod liver oil, and 81 parts of corn-starch. Arachin was selected as the source of protein because of its known low methionine content (13). This mixture was supplied *ad libitum*, the average daily consumption being 15 gm. per rat.

Each rat in Group I received, in addition to the above diet, 24 mg. of guanidoacetic acid and 146 mg. of normal ethanolamine hydrochloride daily, for 1 week. Then, for 3 days, the ethanolamine complement was replaced by an identical amount of isotopic ethanolamine. The mean weight of the three rats was initially 251 gm., and at the end of the feeding, 248 gm.

Each rat in Group II received, in addition to the basal diet, 230 mg. of betaine hydrochloride daily. After 7 days, 146 mg. of isotopic ethanolamine hydrochloride were added to the daily ration for 3 days. The initial mean weight of these rats was 249 gm., and at the end of the experiment, 234 gm.

The test substance in each case was the same ethanolamine hydrochloride previously described (5) and contained 2.00 atom per cent excess N^{15}.

The animals were killed and their organs and carcasses treated precisely as in the previous report (5). Ethanolamine was ultimately recovered as the picrolonate (m.p., 228°, 228°), and choline as the mercuric chloride double salt (m.p., 248–249°, 248–250°).

$C_5H_{14}NOCl_{13}Hg_6$. Calculated, N 0.79; found, N (Kjeldahl) 0.74, 0.84

Glutamic acid was isolated as the hydrochloride.

$C_5H_{10}NO_4Cl$. Calculated, N 7.6; found, N (Kjeldahl) 7.6, 7.8

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[1] Arachin was prepared from a sample of peanut flour supplied through the generosity of Planters Edible Oil Company, Suffolk, Virginia, for which the author wishes to express his gratitude.

Glycine was isolated as the trioxalatochromiate and purified as the *p*-toluenesulfonyl derivative (m.p., 147–148°, 147–148°).

$C_9H_{11}NO_4S$. Calculated, N 6.1; found, N (Kjeldahl) 6.2, 6.1

SUMMARY

Isotopic ethanolamine has been fed to rats on diets with and without an adequate supply of available methyl groups.

From comparison of the ratios of isotopic nitrogen in the ethanolamine and choline isolated from the phosphatides of these animals, it appears that the conversion of ethanolamine to choline proceeds without hindrance in rats even when the diet is sufficiently poor in methyl groups to cause fatty liver.

The author wishes to acknowledge his indebtedness to the late Dr. R. Schoenheimer for his valuable advice and to Dr. D. Rittenberg for his help and cooperation.

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KETOSIS IN PRIMATES

II. THE RATIO OF β-HYDROXYBUTYRIC ACID TO ACETOACETIC ACID IN THE BLOOD AND URINE*

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A study of the data contained in the literature pertaining to the acetone bodies reveals great differences in the relative quantities of β-hydroxybutyric acid and acetoacetic acid present in the blood as compared with that present in the urine. Kennaway (1) was the first to show that the percentage of the total acetone bodies present as β-hydroxybutyric acid in the urine of diabetic human subjects is not constant and that it varies with the degree of ketosis. In his series this acid constituted from 32 to about 84 per cent of the total acetone bodies. Such a trend is shown also in Fig. 1 which contains representative data from diabetic (2–4) and fasting obese subjects (5) and from subjects on a ketogenic diet (6). In contrast to the great fluctuations of the ratio of β-hydroxybutyric to acetoacetic acid in the urine, the blood contains a relatively constant ratio of β-hydroxybutyric acid to acetoacetic acid. Thus in nine samples taken from seven fasting subjects, whose blood contained from 1.25 to 6.15 mM of total acetone bodies per liter of blood, Crandall (7) found the following relative percentages of the hydroxy acid, 60, 62, 68, 60, 80, 62, 59, 61, and 66. The average, 64 per cent, corresponds to a ratio of hydroxy to keto acid of 1.78. From a consideration of these data it is clear that in mild ketosis the ratio of the two acids contained in the urine is smaller than that in the blood; on the other hand, it is greater than that of blood in conditions leading to severe ketosis.

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These data suggest a number of possibilities. Since the ratio is altered by the kidney, the change could be due to a difference in the rate of oxidation of the two acids in the kidney. Snapper and Grünbaum (8, 9) have shown that the perfused kidney possesses an unusual ability to burn acetone bodies. Acetoacetic acid is rapidly metabolized, and a portion is converted into β-hydroxybutyric acid. On the other hand, β-hydroxybutyric acid, although rapidly oxidized, does not apparently yield acetoacetic acid. The results of Snapper and Grünbaum can best be explained by assuming a greater rate of oxidation of acetoacetic acid. Furthermore, since the kidney contains the specific dehydrogenase (10), the ratio of the acids may also be affected by the oxidative conditions present in the glomeruli and tubules. Finally, the possibility of differences in the relative rates of excretion, or of "clearance," of the two acids at various levels of ketosis should be considered.

A study of the ratio of β-hydroxybutyric to acetoacetic acid in blood and urine should have considerable practical value. As is known, the accurate determination of the acetone bodies presents many technical difficulties. Although the method for the determination of acetoacetic acid is relatively simple, the determination of β-hydroxybutyric acid is not only more difficult and time-consuming but also subject to more errors. β-Hydroxybutyric acid must be oxidized to acetone. But the yield of acetone is variable, and many substances, unless removed, may cause large errors in the results. Acetoacetic acid is rapidly decomposed into acetone and CO_2 by amino compounds (11), and considerable quantities may be reduced to β-hydroxybutyric acid by the growth of bacteria in the sample. Acetone is quite volatile. Therefore, a small loss of acetone by volatilization during storage and the subsequent analysis and the reduction of some of the acetoacetic acid by bacterial action greatly alter the ratio of the hydroxy to the keto acid. This would account, perhaps, for the unusually high ratios often found in the literature (see Fig. 1 for example). If it could be shown that fairly constant and reproducible ratios of hydroxy to keto acid are obtained in human subjects under widely different conditions (and that these relations are true as well for other Primates) by eliminating, as far as possible, the errors due to improper handling of samples and faulty analytical technique, then the determination of total acetone bodies would involve

merely a determination of acetoacetic acid. From the ratios so determined, and from determinations of the ratios of the reduced to the oxidized compounds of other oxidation-reduction systems,

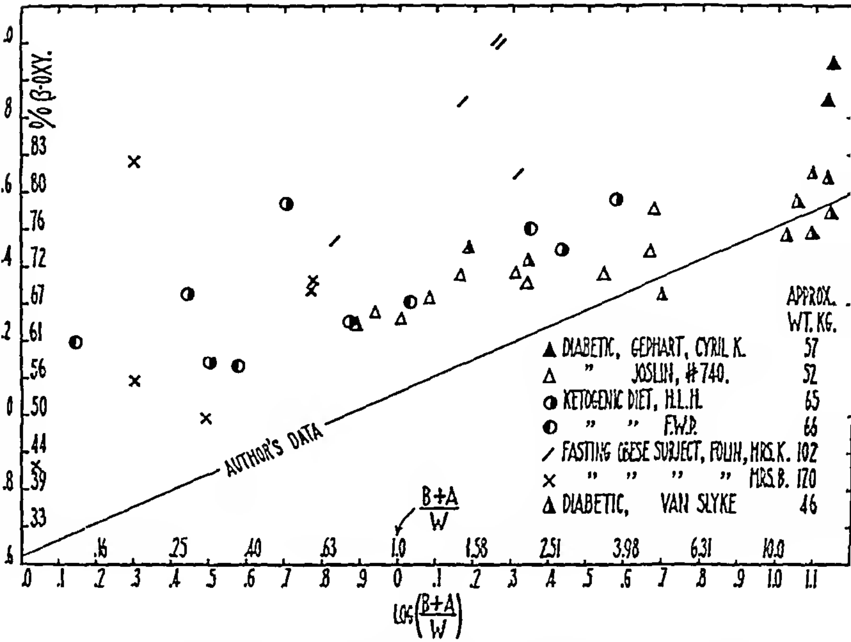

FIG. 1. Ketonuria of human subjects. The data are taken from Gephart, Aub, Du Bois, and Lusk (2), Joslin (3), Van Slyke (4), Folin and Denis (5), and Higgins, Peabody, and Fitz (6). The logarithm of the millimoles of total acetone bodies excreted in 24 hours, $B + A$, per kilo of body weight, W, is represented on the abscissa; the logarithm of the ratio of β-hydroxybutyric, B, to acetoacetic acid, A, is represented on the ordinate.

it would be possible also to gain an insight into the oxidative conditions obtaining in tissues.[1]

EXPERIMENTAL

Blood was collected in cold, sterile syringes and transferred to small, cold specimen bottles containing a minimum of sodium

[1] This point is not considered in this paper.

oxalate. A large sample was immediately precipitated by the Folin-Wu procedure. An aliquot of the clear solution was treated with the Van Slyke $CuSO_4$ and $Ca(OH)_2$ reagents. The acetone bodies were then determined by a modification of the Shaffer-Marriott method (12). Throughout, every effort was made to minimize the loss of acetone by volatilization. Thus the precipitations were carried out in the cold and the solutions were cleared by centrifugation, instead of filtration, in the cold. All measurements of samples of aliquots were made with cold pipettes.

Urine was collected in cold bottles containing 5 cc. of washed[2] toluene. Analyses were made as soon as possible after the end of the period of collection. As in the case of blood, all operations, including measurements of samples and aliquots, and the precipitations and centrifugations, were carried out in the cold.

The animals were healthy *rhesus* monkeys. They were placed in small metabolism cages. Although the space was limited, they were nevertheless very active. The consumption of water was increased after the onset of ketosis. The animals frequently urinated and the volume of urine was considerably greater than normal. The specimen bottles below the cages were changed at intervals of 4 hours.

Results

Fasting Ketosis in Monkeys—The results from five animals, three males and two females, showed great individual differences (Table I). Acetone bodies appeared in the urine within the first 24 hours. Monkey 28, a small, tame, immature animal, attained the maximum ketosis on the 2nd day. The intensity of the ketosis at this time was greater than that attained by any of the other animals. A total of 27.4 mM, or 11.4 mM per kilo, was excreted in 24 hours. The ketosis was still as intense on the 3rd day, and it rapidly declined on the 4th day. In the other four animals the ketosis progressed more slowly, reaching its highest level on the last day of the fast. However, the rate of development of ketosis differed greatly.

[2] C.P. toluene contains variable quantities of water-soluble substances yielding iodoform with hypoiodate under the conditions of the Messinger titration. The commercial or "technical" grades may contain very large quantities of iodine-binding substances, probably acetone.

In a study of the fasting metabolism of five men and five women, Deuel and Gulick (13) without exception noted that the acetonuria was more slowly developed and the rate of excretion attained on the 4th day of fasting was smaller in the men than in the women.

TABLE I

Fasting Metabolism of Five Macacus rhesus Monkeys

Monkey No.	Sex	Weight before fast	Day of fast	Total urinary N, calculated to 24 hrs.	Acetone bodies in urine, calculated to 24 hrs.		
					β-Hydroxybutyric acid (*B*)	Aceto-acetic acid (*A*)	Total (*B*) + (*A*)
		kg.		*gm.*	*mM*	*mM*	*mM*
28*	Male	2.4	2nd†	0.91	22.0	5.4	27.4
			3rd	0.89	21.0	6.2	27.2
			4th	0.87	7.2	3.3	10.5
31	Female	3.7	2nd†	0.97	1.4	0.3	1.7
			3rd	1.18	3.4	2.0	5.4
			4th	1.24	4.0	2.6	6.6
32‡	Male	4.0	2nd†	1.80	4.4	3.1	7.5
			3rd	1.79	8.8	4.1	12.9
			4th	3.57	22.1	8.5	30.4
34	Female	4.0	2nd†	1.30	2.2	2.2	4.4
			3rd	1.23	6.5	2.6	9.1
			4th	1.61	7.9	3.1	11.0
35	Male	6.1	2nd†	2.18	0.56	0.95	1.51
			3rd	2.11	0.83	1.33	2.16
			4th	2.40	1.27	1.75	3.02
35	"	5.4	2nd§		0.84	0.99	1.83
35	"	6.1	2nd‖		0.62	0.83	1.45
			3rd		1.07	1.40	2.47
			4th		1.32	1.73	3.05

* Small; immature as indicated by teeth; quite tame.

† First period of fasting, May 24–27, 1931.

‡ An aggressive animal.

§ Second period of fasting, March 29–31, 1933.

‖ Third period of fasting, April 18–21, 1933.

Male rats and guinea pigs also excreted fewer acetone bodies than females (14). In our animals, just the opposite trend was noted. The two males, Monkeys 28 and 32, developed a ketosis more rapidly, and the maximum excretion was greater than that of the

two females, Monkeys 31 and 34. On the other hand, Monkey 35, a male, excreted very small quantities of acetone bodies, less than the two females. In the previous series, the males of each genus had a slightly greater ketonuria than the females. However, again a male animal, a mandrill, proved to be the exception. On the whole, the male monkeys appeared to be more susceptible to the development of a severe ketosis than the females.

That the fasting ketosis of monkeys, as measured by the rate of excretion of acetone bodies, can be as great as the severe diabetic ketosis in human subjects, was first noted by the writer (15). While it is true that the animals previously studied were not normal, the animals used in the present study were in excellent health. In the previous series, the maximum rate of excretion per kilo of body weight was as follows: brown capuchin monkey (*Cebus capucinus*), male, 9.2 mM; black ape (*Cynopithecus niger*), male, 5.4 mM; black ape, female, 5.0 mM; bonnet macaque (*Pithecus sinicus* Desmarest), male, 6.3 mM; bonnet macaque, female, 4.6 mM; mandrill (*Papio sphinx* L.), male, 0.36 mM. In the present series with *Macacus rhesus*, the rates of excretion were as follows: Monkey 28, male, 11.4 mM; Monkey 32, male, 7.6 mM; Monkey 35, male, 0.49 mM; Monkey 31, female, 1.8 mM; Monkey 34, female, 2.8 mM. It is apparent that the maximum excretion of acetone bodies per kilo of body weight was as great in the present as in the former series.

These data should be compared with those from human subjects shown in Figs. 1 and 2. The rate of excretion per kilo of body weight of the two obese fasting subjects and two subjects on a ketogenic diet varied from 0.2 to a maximum of 3.7 mM. The maximum rates of excretion in diabetic human subjects were as follows: Van Slyke's patient, 17.4 mM; Cyril K. (3), 14.2 mM. These data again demonstrate the great ease with which a severe ketosis is attained in monkeys. Because of their small size, and therefore greater metabolism per kilo of body weight, the available glycogen stores of monkeys are, perhaps, more rapidly exhausted than in adult human subjects. This factor and the intense acidosis may account for the unfavorable effect of a few days of fasting (weakness and coma) observed in six monkeys of the first series and one monkey of the present series.

Ratio of β-Hydroxybutyric to Acetoacetic Acid in Blood—Table II

contains data from three depancreatized dogs and five human subjects. Blood was collected from the human subjects under relatively uniform conditions, either after they had been in bed or

TABLE II

Acetone Bodies in Blood

Subject	Comment	Time of collection	Condition of subject	Blood		
				β-Hydroxybutyric acid (B)	Acetoacetic acid (A)	$\frac{B}{A}$
				mM per l.	*mM per l.*	
Dog 1	Depancreatized			0.40	0.21	1.90
" 2	"			0.42	0.74	1.92
" 3	"		Moribund	1.97	1.00	1.97
Human, Mr. G.	Ketogenic diet	Feb. 1, 8.30 p.m.	Asleep	1.02	0.56	1.82
" " "	" "	Feb. 1, 9.00 p.m.	Awake	1.31	0.66	1.98
" Mrs. H.	" "	Feb. 7, 3.00 p.m.	Out-patient clinic	3.65	1.96	1.86
" " "	" "	Feb. 21, 4.00 p.m.	" "	2.51	0.93	2.70
" Mr. W.	" "	Feb. 8, 2.00 p.m.	" "	1.73	0.90	1.93
" " "	" "	Feb. 8, 5.00 p.m.	" "	2.01	0.87	2.31
" Mr. C.	Diabetic	Apr. 3, 10.00 p.m.	Asleep	4.32	2.68	1.61
" " "	"	Apr. 4, 10.00 a.m.	Ambulatory	4.45	2.08	2.14
" Miss W.	See Table IV	May 1, 8.30 p.m.	Resting in bed	9.17*	5.17*	1.77

* The plasma was analyzed. The blood was centrifuged in the cold immediately after collection in a cold, stoppered centrifuge tube.

had been sitting in an easy chair for some time. Blood was obtained from dogs which had been trained to lie quietly. Blood, unlike the urine, contained a fairly constant relative proportion of

the two acids over a wide range of concentration of total acetone bodies. Surprisingly, the ratio was about the same in dog (1.93) as in human blood (2.01). Greater variations of the ratio, from 1.43 to 4.0, and a somewhat lower average ratio of 1.78 were observed by Crandall.

The variations in the ratio cannot be ascribed entirely to analytical errors. To some extent they may be caused by differences in activity. The higher results obtained from untrained dogs in Crandall's series may be accounted for by this means. In the case of subject G in the writer's series, the ratio of β-hydroxybutyric to acetoacetic acid in the blood was smaller at the time of awakening at 8.30 p.m. than half an hour later. In the case of the diabetic subject C, the ratio was 1.61 when the patient was aroused at 10 p.m., and it was 2.14 at 10 a.m. the next day when the patient came to the laboratory. Subjects H and W, clinic patients, had been undergoing tests and treatments since about 10 o'clock on each of the days indicated, and it is significant that the blood taken late in the afternoon in each instance contained a high relative quantity of β-hydroxybutyric acid.

Ratio of β-Hydroxybutyric to Acetoacetic Acid in Urine—Data from three human diabetic patients and one very fat, healthy subject are shown in Fig. 2, together with data from the total of 19 days of fasting of the five monkeys. Although the results are plotted as in Fig. 1, the basis of comparison differs in many important respects. Thus, results from human subjects (Tables III to V), varying in age from 15 to 44 years and weighing from 40 to 129 kilos, are compared with those from monkeys, varying perhaps just as greatly as to age but weighing from 2.4 to 6.1 kilos.

The condition of the diabetic subjects varied greatly from the normal. Mrs. P. H. (Table III) had many large abscesses, and her temperature varied from 38.3–39.4°. She received large volumes of Ringer's solution and 0.9 per cent NaCl solution on the previous day, which was the day of admission, and on the day the collection of samples was begun. A total of 160 units of insulin was given in three doses at the times shown in Table III. The data are shown in Fig. 2. Mrs. L. M. (Table IV) was considerably undernourished; otherwise, she was quite normal. Miss W. (Table V), aged 15 years, complained of fatigue, sleeplessness, and shortness of breath. Only one specimen of urine was collected.

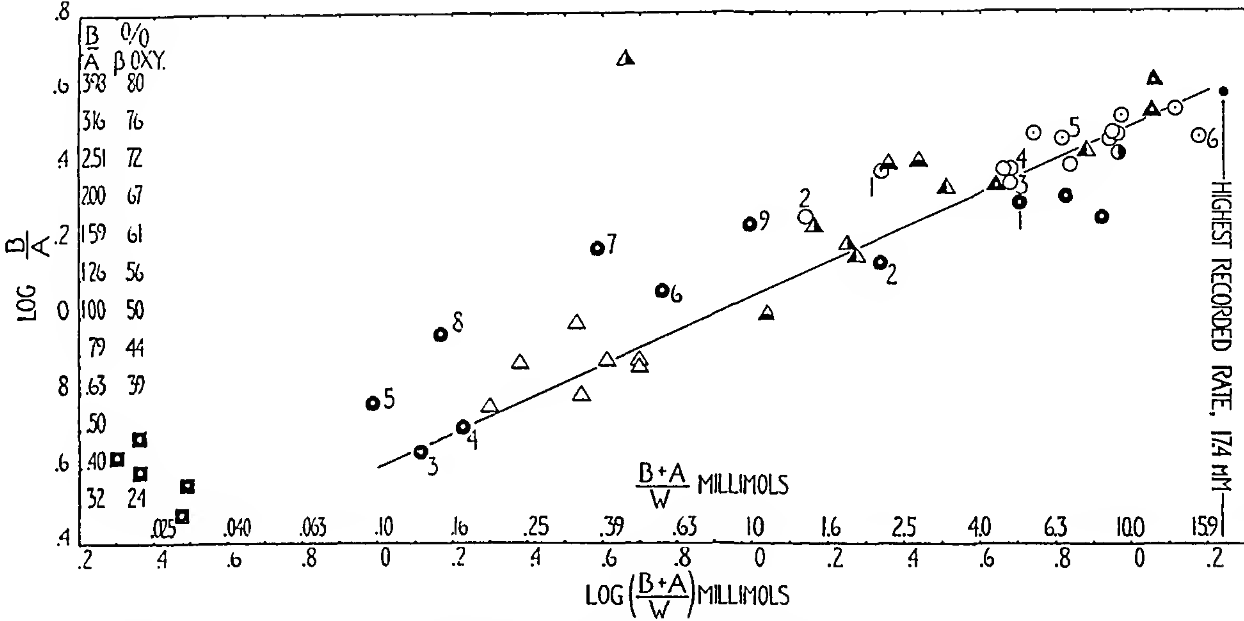

FIG. 2. Ketonuria of monkeys and human subjects. Data from monkeys are indicated by triangles; from three diabetic human subjects by circles; and from one human subject on a ketogenic diet by squares. (Mrs. P. H., solid circle; Mrs. L. M., clear circle; Miss W. circle shaded one-half.) The numbers indicate successive samples collected after the administration of insulin. The basis of comparison is the same as in Fig. 1. The heavy dot in the upper right corner represents the highest result from Van Slyke's diabetic subject.

The result is indicated in Fig. 2. Miss R., who weighed 129 kilos, received a ketogenic reducing diet. Within a few days a slight ketonuria, which varied from 2.15 to 3.94 mM in 24 hours, developed.

The numbered points in Fig. 2, which represent the data from the periods following the injection of insulin, account for most of the large deviations. But the variations are not nearly as great as those in Fig. 1. The curve, a straight line drawn through the lower data, is at a lower level than in Fig. 1. The writer cannot, of course, say whether this is due to close attention to analytical technique or to the precautions taken to prevent loss of acetone and conversion of acetoacetic acid to β-hydroxybutyric acid. It should be noted that Van Slyke's data fall within the range of the writer's data.

TABLE III

Diabetic Human Subject

Mrs. P. H.; age 44 years; height 154 cm.; weight 65.3 kilos, intake of fluids 4025 cc.; date, August 25, 1935.

Date	Urine collection period	Volume of urine	Insulin	Acetone bodies in urine, calculated to 24 hrs.		
				β-Hydroxybutyric acid (*B*)	Aceto-acetic acid (*A*)	Total acetone bodies (*B*) + (*A*)
Aug., 1935		*cc.*	*units*	*mM*	*mM*	*mM*
25	5.10 p.m.– 9.10 p.m.	745		348	194	542
25	9.10 " – 1.10 a.m.	692		293	144	437
26	1.10 a.m.– 5.10 "	660	80, 1.30 a.m.	218	112	330
26	5.10 " – 9.45 "	718	40, 7.50 "	82	60	142
26	9.45 " – 1.07 p.m.	183	40, 10.50 "	2.5	5.7	8.2
26	1.07 p.m.– 3.22 "	283		3.6	7.1	10.7
26	3.22 " – 6.52 "	278		2.3	3.9	6.2
26	6.52 " –10.47 "	510		20.3	17.7	38.0
26	10.47 " – 2.47 a.m.	300		15.2	10.2	25.4
27	2.47 a.m.– 7.21 "	293		4.5	5.0	9.5
27	7.21 " – 8.20 "	336		40.6	23.6	64.2

The results demonstrate beyond question that the ratio of β-hydroxybutyric acid to acetoacetic acid in the urine is a function of the rate of excretion of the total acetone bodies. The ratio of the

two acids increases as the rate of excretion increases; the blood, on the other hand, contains a relatively constant ratio of the ketone acids over a wide range of concentrations. It is evident that the two acids are excreted or "cleared" at different rates by the kidney. This is illustrated by the data obtained from Miss W., a diabetic patient, who, over a period of 2 hours, from 7.30 to 9.30 p.m., excreted total acetone bodies at the rate of 9.65 mM per kilo per

TABLE IV

Diabetic Human Subject

Mrs. L. M.; age 23 years; height 163 cm.; weight 40.3 kilos on June 23, 1931.

Date	Urine collection period	Volume of urine	Acetone bodies in urine, calculated to 24 hrs.		
			β-Hydroxybutyric acid (*B*)	Acetoacetic acid (*A*)	Total (*B*) + (*A*)
June, 1931		*cc.*	*mM*	*mM*	*mM*
23	4.30 p.m.- 4.30 a.m.	1410	271	91	362
24	4.30 a.m.- 4.30 p.m.	1355	290	88	378
24	4.30 p.m.- 8.30 "	630	405	118	523
25	8.30 " - 4.00 a.m.	560	165	56	221
25	4.00 a.m.- 7.30 "	295	132	55	187
25	7.30 " -10.30 "	420	267	93	360
25	10.30 " -11.30 "	120	274	93	367
25	11.30 " - 3.30 p.m.*	290	197	80	277
25	4.30 p.m.- 8.45 "	160	62	26	88
25	8.45 " - 7.30 a.m.	480	36	20	56
26	7.30 a.m.-10.30 "	400	132	60	192
26	10.30 " -11.30 "	100	132	55	187
26	11.30 " - 1.15 p.m.	170	195	68	263
26	1.15 p.m.- 3.30 "	385	435	150	585

* 40 units of insulin at 3.30 p.m.

24 hours. The plasma at 8.30 p.m. contained 14.34 mM of total acetone bodies per liter. At a rate of excretion of 2.96 cc. of urine per minute, the urine contained 5.80 times as much acetoacetic acid and 8.62 times as much β-hydroxybutyric acid as the plasma.

The maximum clearances, when calculated by the formula,

$$\text{Clearance} = \frac{\text{concentration in urine} \times \text{cc. urine excreted per minute}}{\text{concentration in plasma}}$$

of Möller, McIntosh, and Van Slyke (16), were as follows: acetoacetic acid 17, β-hydroxybutyric acid 26, total acetone bodies 23. Crandall noted ratios of total acetone bodies of urine to blood of normal fasting human subjects varying from 7.7 to 22.8, with an average ratio of 14.9. Assuming an average rate of excretion of urine of 1 cc. per minute, or 1440 cc. in 24 hours, such a ratio would correspond to a clearance of 15. It is evident that the acetone bodies contained in the glomerular filtrate are readily reabsorbed in the tubules.

As already stated, the ratio of hydroxy to keto acid in the urine is probably determined by such factors as the oxidation-reduction conditions in the kidney, the relative rates of oxidation of the acids

TABLE V

Acetone Bodies in Plasma and Urine of Diabetic Patient, Miss W.

355 cc. of urine were excreted from 7.30 to 9.30 p.m. The rate of excretion per minute, V, was 2.96 cc. Blood was taken at 8.30 p.m.

	Plasma concentration per liter (P)	Urine		Clearance $\frac{UV}{P}$
		Concentration per liter (U)	Calculated excretion in 24 hrs.	
	mM	mM	mM	
β-Hydroxybutyric acid	9.17	79	336	26
Acetoacetic acid. ...	5.17	30	128	17
Total acetone bodies	14.34	109	464	
β-Hydroxybutyric acid / Acetoacetic acid . .	1.77	2.63		

by the kidney, and the relative rates of reabsorption of the acids in the tubules. The effect of these various factors cannot be inferred from the data given in the present paper. However, the results should have practical analytical application.

The writer gratefully acknowledges the interest and help given in this work by Dr. Russell M. Wilder, Dr. H. B. van Dyke, Dr. Louis Leiter, and Dr. Henry T. Ricketts in providing clinical material for this study.

SUMMARY

Five healthy *rhesus* monkeys, three male and two female, weighing from 2.4 to 6.1 kilos, were fasted for periods of 2 to 4 days.

The excretion of acetone bodies in the urine of four human subjects, three diabetic and one obese subject receiving a ketogenic diet, was also studied. Two of the diabetic subjects received insulin during the period of observation. The age of the subjects varied from 15 to 44 years and the weight from 40 to 65 kilos.

With one exception, the monkeys developed a ketosis within 2 days which was as intense as that previously obtained in monkeys afflicted with "cage paralysis." Male monkeys appeared to be more susceptible to the development of severe ketosis than the females. This is contrary to the conclusions of previous workers who experimented with human subjects, rats, and guinea pigs.

The ratio of β-hydroxybutyric to acetoacetic acid in the urine is a function of the rate of excretion of total acetone bodies per kilo of body weight. It varies from approximately 0.5 at the "threshold" of ketosis to approximately 4 in very severe diabetic ketosis of human subjects. At the same rate of excretion per kilo of body weight, the ratio of the two acids is the same in the urine of monkeys as in human subjects. All of the results fall within a narrow range, owing perhaps to the close attention to analytical technique and to precautions taken in sampling.

In ketosis from various causes, the ratio of hydroxy to keto acid in the blood of three dogs and five human subjects (twelve samples) varied from 1.61 to 2.70, with an average of 1.99.

The factors responsible for these differences between the ratio of hydroxy to keto acid in blood and urine are briefly discussed and the practical analytical applications are pointed out.

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THE ISOLATION OF ALLOPREGNANOL-3(β)-ONE-20 FROM HUMAN PREGNANCY URINE*

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Although allopregnanol-3(β)-one-20 has been isolated from the urine of pregnant sows (1) and pregnant mares (2, 3), it has not heretofore been reported in human pregnancy urine. Other natural sources of this steroid are the corpus luteum (4–6) and the adrenal gland (7).

The pregnancy urine investigated was obtained during labor and delivery. The urine was brought to pH 1 with hydrochloric acid and exhaustively extracted with dibutyl ether at 90°.[1] The ketonic material obtained from the neutral extract by the use of Girard's Reagent T (8) was allowed to react with succinic anhydride in order to obtain alcoholic and non-alcoholic fractions (9). The hydroxy ketones were treated with 70 per cent methanol containing 2 per cent digitonin and the insoluble digitonides were worked up by the usual procedures. There were obtained, in this manner, 134.5 mg. of a brown oil, constituting the digitonin-precipitable hydroxy ketones of 133 liters of urine. When the oil was refluxed for 2 hours in a 90 per cent alcoholic solution of semicarbazide acetate and cooled to room temperature, 57.7 mg. of a crystalline semicarbazone, m.p. 238–242°,[2] were obtained. Hydrolysis, which was effected by refluxing in 0.5 N hydrochloric acid in 80 per cent alcohol, yielded 44.4 mg. of white crystalline material. When crystallized from absolute methanol, 14.4 mg. of plates, m.p.

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[1] The hydrolysis and extraction were carried out in the Research Laboratories of the Schering Corporation, Bloomfield, New Jersey, through the courtesy of Dr. E. Schwenk.

[2] All melting points are corrected.

175–178°, $[\alpha]_D^{20}$ + 70° (0.40 per cent in absolute ethanol) were obtained. Two more recrystallizations from the same solvent raised the melting point to 187–188°; another recrystallization gave 3.3 mg., melting sharply at 189–190°. Admixture with an authentic specimen of allopregnanol-3(β)-one-20, m.p. 191°, which

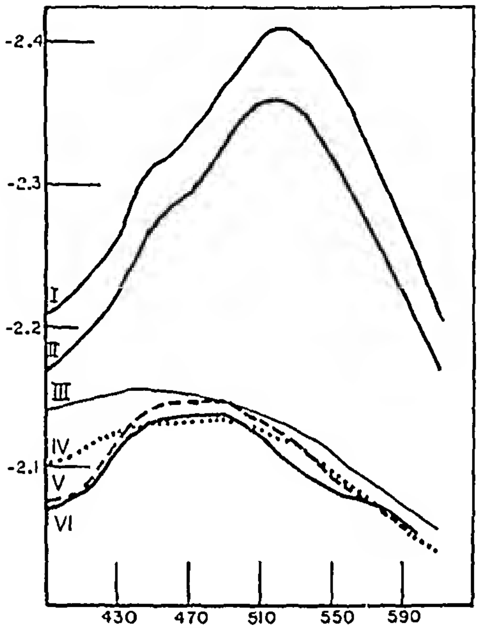

Fig. 1. Absorption spectra (Zimmerman test) of steroids derived from the digitonin-precipitable hydroxy ketonic fraction. The abscissa scale represents wave-length in mμ; the ordinate, optical density (log 1/transmission). Curve I, synthetic dehydroisoandrosterone (for comparison). The following indicate crystalline mixtures: Curve II, m.p. 150–165°; Curve III, m.p. 152–154°; Curve IV, m.p. 155–159°; Curve V, allopregnanol-3(β)-one-20, m.p. 189–190°; Curve VI, allopregnanol-3(β)-one-20, m.p. 191° (from corpus luteum for comparison). Curves I and II are for 50 γ and Curves III to VI for 100 γ of crystalline steroid each. Note that Curve III is unique in that the absorption maximum is at 430 to 440 mμ and is quite high from 390 to 420 mμ.

was kindly furnished by Professor O. Wintersteiner, gave no depression in melting point. The absorption spectra in a modified Zimmerman test (10) were almost identical for both specimens (Fig. 1, Curves V and VI). The oxime derivatives of both specimens melted at 222–223° and gave no mixed melting point depres-

sion. An additional 4.0 mg. of allopregnanol-3(β)-one-20, m.p. 187–188°, were obtained from the mother liquors; the acetate derivative melted at 139–140°, whereas the acetate of the authentic specimen, which had been prepared simultaneously, melted at 138–140° and gave no mixed melting point depression.

When the remainder of the digitonin-precipitable hydroxy ketones was subjected to chromatographic analysis, only small amounts of crystalline mixtures could be obtained; the melting points and absorption curves (Zimmerman test) of these are given in Fig. 1. Curve IV (Fig. 1) is that of a C_{20} ketosteroid mixture. Such a mixture might have as a component the C-17-stereoisomer of allopregnanolone, a minor percentage of which is known to arise as the result of alkali treatment of the normal steroid (11). The subsequent acid treatment in the hydrolysis of the semicarbazones may not have reversed this isomerism. It is also possible that pregnanol-3(β)-one-20 is a minor constituent of labor and delivery urine, since the C_3 epimer of this steroid as well as that of allopregnanol-3(β)-one-20 has been reported by Marker *et al.* (12, 13) to occur in human pregnancy urine. Δ^5-Pregnenol-3(β)-one-20 which has been suggested by Butenandt (14) as a probable intermediate in the formation of progesterone would be found in the digitonin-precipitable hydroxy ketonic fraction if indeed present. The 17-ketosteroids found in the corresponding fraction of non-pregnant female urine were not isolated probably because of the complexity of this fraction in pregnancy urine and the small amount of available material. That 17-ketosteroids are not absent is indicated (Curve II, Fig. 1); the presence of ketones other than the C_{20} and C_{17} varieties is also indicated (Curve III). The new androstanol-3(β)-one (?) reported by Heard and McKay (3) to occur in pregnant mare urine failed to be isolated. Studies of late human pregnancy urine are in progress in order to examine more completely this fraction in the light of the foregoing suggestions and especially to see whether allopregnanol-3(β)-one-20 is characteristic only of labor and delivery.

SUMMARY

Allopregnanol-3(β)-one-20 is a constituent of human labor and delivery urine. The presence of limited amounts of 17-ketosteroids in the digitonin-precipitable fraction is indicated.

We are very much indebted to the obstetrical staffs of the Worcester City Hospital and the St. Vincent's Hospital of Worcester for cooperation in the collection of the urine specimens. Professor O. Wintersteiner was extremely kind in placing the facilities of his laboratory at our disposal for a portion of the work.

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THE INFLUENCE OF SUPPLEMENTARY CASEIN, CYSTINE, AND METHIONINE ON LIVER LIPID CONTENT

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It has been our experience that a lipotropic action was observed almost universally whenever cystine and methionine were added to a low protein (5 per cent), high fat (40 per cent) diet in amounts sufficient to raise the quantities of the sulfur-containing amino acids to the same levels as those of rations containing from 18 to 20 per cent casein and 40 per cent lard (1, 2). The lipotropic action observed when the total amino acid content of diets was increased by merely elevating the protein content was attributed by us to the increase in the dietary methionine (1). Other workers in this field have, however, presented data which led them to conclude that the lipotropic activity of dietary proteins cannot be explained solely on the basis of their cystine and methionine content. According to Channon and coworkers (3) "added methionine is incapable of exerting its full action in the absence of some other protein constituents." Best and Ridout (4) support this conception by their observation that the feeding of a basal diet (5 per cent meat powder, 40 per cent fat) supplemented with amounts of cystine and methionine equivalent to those present in a ration containing 30 per cent casein as the sole source of protein exerted virtually no effect on the fat content of the livers. This was in contrast to the decided lipotropic action observed when the same basal diet was supplemented with 30 per cent casein.

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In the light of these divergent opinions it seemed desirable to obtain additional information on this question. The experimental and analytical procedures employed (1) were essentially the same as those used by the other investigators referred to above. All diets contained 2 per cent agar, 40 per cent lard, and 5 per cent salt mixture (5). To these were added casein and the amino acids as supplements in the amounts specified in Table I. The remainder of the rations consisted of glucose. Each rat received one yeast tablet (500 mg.) and 2 drops of cod liver oil daily. The cystine and methionine contents of the rations given in Columns 5 and 6 of Table I represent the sums of the amounts present in the casein (6) and the supplementary amino acids.

Diets 1 to 7 inclusive contained an amount of methionine (620 mg. per cent) equivalent to that present in a ration containing 20 per cent casein as the sole source of protein. The progressive addition of larger amounts of cystine to these diets was without influence until a level of 817 mg. (Diet 6) was reached and then only a slight elevation of liver lipids (15.7 per cent total lipids) was observed. At the 1017 mg. level (Diet 7) distinctly fatty livers were obtained. Thus approximately 15 times the amount of cystine present in a 20 per cent casein diet was required to overcome the lipotropic action of the 620 mg. per cent of methionine. This is the amount of methionine contained in a 20 per cent casein diet.

The ability of methionine to overcome the effect on liver lipid content of 600 mg. per cent of supplementary cystine was determined by feeding Diets 9 to 12 inclusive. The 23.6 per cent of liver lipids reported for the rats on Diet 8 is the amount usually observed after feeding a low protein-high fat diet. The data on the animals on Diet 11 demonstrate that a diminished liver lipid value was observed when the methionine content was raised to 465 mg. per cent. The increase of total methionine from 310 to 465 mg. per cent caused a decrease of approximately 42 per cent of liver fat content. Diets 13, 14, and 15 are respectively like Diets 10, 11, and 12 as far as methionine content is concerned, except that in the case of the first three the quantity of methionine in the diets was increased by adding casein rather than by the free amino acid. A comparison of the data obtained shows that a 42 per cent drop in liver fat content occurred when the methionine level was raised

from 310 mg. per cent (Diet 10) to 465 mg. per cent (Diet 11). In both instances the basal diet was supplemented with the amino

TABLE I

Influence of Supplementary Casein, Cystine, and Methionine on Liver Lipid Content

The figures in parentheses are the values for the individual rats. The length of each experimental period was 21 days. Unless otherwise stated the group fed each diet included eight rats.

Diet No.	Supplements in 100 gm. of diets			Sulfur-containing amino acids in 100 gm. of diets		Average daily food intake	Change in weight	Liver lipids
	Casein	Cystine	Methionine	Cystine	Methionine			
	gm.	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*per cent*	*per cent in liver*
1	0	0	465	17	620	6.6	+25	11.8 (7.9-15.9)
2	0	100	465	117	620	6.6	+17	11.6 (8.9-16.9)
3	0	200	465	217	620	6.7	+22	11.2 (7.0-15.0)
4	0	400	465	417	620	7.4	+13	14.0 (7.2-18.5)
5	0	600	465	617	620	6.0	+10	12.0 (8.9-15.2)
6	0	800	465	817	620	6.2	+9	15.7 (11.6-20.3)
7*	0	1000	465	1017	620	6.7	+14	19.7 (13.3-30.3)
8	0	0	0	17	155	6.0	−5	23.6 (15.9-34.3)
9	0	600	0	617	155	5.7	+18	35.1 (28.2-42.7)
10*	0	600	155	617	310	6.1	+24	32.8 (27.3-38.4)
11	0	600	310	617	465	6.3	+31	19.0 (13.4-28.3)
12	0	600	620	617	775	5.6	+18	9.9 (5.8-13.0)
13†	5	583	0	617	310	‡	+28	27.1 (18.2-37.7)
14†	10	566	0	617	465	‡	+38	30.3 (24.6-38.1)
15	20	532	0	617	775	‡	+46	19.8 (14.6-30.4)
16	15	49	0	117	620	9.4	+45	13.9 (7.5-18.2)
17	15	149	0	217	620	9.0	+47	26.3 (22.2-32.4)
18	15	349	0	417	620	9.0	+41	20.6 (13.0-28.4)
19	15	749	0	817	620	8.8	+49	26.5 (21.6-33.0)
20	15	949	0	1017	620	9.0	+43	22.4 (18.5-28.8)

* Seven animals.

† Six animals.

‡ These animals ate practically all of the 10 gm. of food given them daily.

acid itself. When casein was used as the supplement (Diets 13 and 14), no fall in liver lipids took place. In fact a slight increase was observed. The values for the livers of the rats fed Diets 11

and 12 and Diets 14 and 15 show that a rise in the methionine level from 465 to 775 mg. per cent was accompanied by a drop in liver fat content of 48 per cent when the methionine was added as such and a 35 per cent fall when casein was added. These data also fail to support the view that the protein, casein, is superior to the amino acid so far as lipotropic activity is concerned. A further consideration of the data reveals the fact that even though the liver fat content of the rats on Diet 15 was 35 per cent lower than that of the animals on Diet 14 the livers of the former group were still distinctly fatty (19.8 per cent fat) as compared with the almost normal liver fat (9.9 per cent) for the animals on Diet 12.

In Diets 16 to 20 inclusive all of the methionine was present as casein, whereas in Diets 1 to 7 listed in Table I only 155 mg. per cent were present in this form. The data secured from the rats on these diets demonstrate that free methionine is very efficient in preventing the usual fatty infiltration caused by the addition of cystine to low protein-high fat diets (7), since the 465 mg. per cent of methionine added to the basal diet practically prevented this particular cystine effect, even though as much as 817 mg. per cent of cystine was present in Diet 6. On the other hand distinctly fatty livers (26.3 per cent lipids) were observed when Diet 17 which contained only 217 mg. per cent of cystine was fed. The amount of methionine present in this latter diet was the same as that of Diet 6, but was provided entirely by the dietary casein.

Previous work from this laboratory (2) showed that whereas the 20 per cent of casein in a 40 per cent fat diet exhibited marked lipotropic action no such response was demonstrable when the casein level was reduced to 15 per cent. Diet 11 contained the same amount of methionine as a 15 per cent casein diet, but in the case of the former two-thirds of the methionine was present as the free amino acid. A comparison of the data obtained with the animals on Diets 9 and 11 shows the lipotropic effect of the latter ration. In this connection it should be pointed out that this latter diet was effective, even though 617 mg. per cent of cystine (12 times the amount contained in an unsupplemented 15 per cent casein diet) were present.

None of the data lends support to the contention that methionine is incapable of exerting its maximum effect on liver fat content in the absence of other protein constituents. While they may be in-

terpreted as demonstrating that supplementary methionine exhibits a greater lipotropic action when given as the free amino acid than when in combination as protein, a further study of the data may afford an alternative explanation. It is clear from Table I that the average daily food intakes of the animals on the increased protein levels (Diets 13 to 20 inclusive) were practically 50 per cent greater than those of the other animals. An examination of Table I shows that the average gain in weight for the whole experimental period (21 days) of all the animals on the diets containing only 5 per cent of casein was only 16.6 per cent in comparison with the average gain of 42.7 per cent for all of the other animals. Thus the amount of newly formed tissues synthesized by the latter group far exceeded that of the former. It follows, therefore, that larger amounts of methionine were probably required for the additional amounts of tissue protein built up, and, because of this need, less methionine may have been available for lipotropic action. In the light of this possibility a definite conclusion from the work presented herein must be withheld. It would be of interest to repeat this work on adult rats in which the growth factor is practically ruled out. This is now being done in this laboratory.

SUMMARY

Cystine was added in increasing amounts to a low casein (5 per cent), high lard (40 per cent) basal diet supplemented with sufficient amounts of methionine to make the total methionine content equivalent to that of a ration containing 20 per cent casein as the sole source of protein. No substantial increase in the liver lipid content of young rats (120 gm.) was observed until a level of 1017 mg. per cent of total cystine was reached.

Under the conditions obtaining in this investigation diets supplemented with methionine as the free amino acid were, so far as lipotropic action is concerned, superior to the diets containing equivalent amounts of methionine in the form of the protein, casein.

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THE SO CALLED ETHER-INSOLUBLE PHOSPHOLIPIDS IN BLOOD AND TISSUES*

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Ever since it was introduced by Altmann in 1889 (1), acetone has been widely and successfully used for isolating and purifying the phospholipids. However, acetone alone is unable to bring about a complete precipitation of the phospholipids, especially from mixtures with fat and cholesterol. In 1910 Nerking (2) pointed out that the addition of a little magnesium chloride to the acetone made the precipitation of the phospholipids practically complete. Numerous workers since that time have confirmed the fact that acetone and magnesium chloride precipitate all phosphorus compounds soluble in alcohol and ether, even though they may not all be lipids.

While developing his oxidative micromethod, Bloor (3) discovered that phospholipid, prepared from tissues, was insoluble in dry ether after it had been precipitated by acetone and magnesium chloride. If, however, the ether was saturated with water, the phospholipid slowly but completely dissolved. Later Kirk, Page, and Van Slyke (4) observed that, even though they adhered strictly to the directions prescribed by Bloor, some of the phospholipid, from both tissues and blood, did not redissolve in moist ether. They analyzed some of the ether-insoluble material; "The N:P ratio found was 0.875 (instead of the usual ratio for cephalin and lecithin of 0.438), showing this fraction to be a diaminomonophosphatide."

On the basis of this observation, Kirk (5) later developed a method for the determination of the ether-insoluble phospholipids in blood and tissues. And, although careful to point out that the

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ether-insoluble phospholipids did not consist entirely of sphingomyelins, he felt sure that the latter, if present, were included in this fraction. The lecithins and cephalins were believed to be extracted by the moist ether. Using the Roman method, Kirk determined the lecithins by their choline content and estimated the cephalins by difference. Kirk (6) has applied his method in an extensive study of the comparative amount of the three types of phospholipids, lecithins, cephalins, and ether-insoluble phospholipids, in normal and pathological conditions. Dziemian (7) has used the Kirk procedure for the study of the lipids in the red cells of various animals.

Meanwhile Folch and Van Slyke (8) and Christensen (9) have demonstrated that a considerable part of the nitrogen in the petroleum ether-soluble fraction of the alcohol-ether extract of blood is non-lipid in nature, much of it being urea. It is obvious therefore that the N:P ratio in such extracts is of no value as an index of the nature of the lipids present.

About 2 years ago the Kirk procedure[1] was applied to a study of the changes in the lecithin, cephalin, and sphingomyelin contents of blood in conditions in which pronounced changes of the total phospholipid had been observed. With fairly pure brain sphingomyelin, we confirmed the finding that sphingomyelin was completely insoluble in moist ether after precipitation by acetone and magnesium chloride. When the method was applied to extracts of blood plasma, there was excellent agreement between duplicates and the relative proportions of the lecithins, cephalins, and sphingomyelins in blood plasma were more uniform from one individual to another than had been found by Kirk (6).

In due time, the solution of magnesium chloride had to be replenished. For several successive analyses the concentration of the solution used increased from one analysis to another. In these analyses, higher and much less consistent values for the ether-insoluble phospholipids in blood plasma were obtained instead of the low and uniform values found previously. We were led to suspect, therefore, a relationship between the percentage of ether-insoluble phospholipids and the amount of magnesium chloride added to the acetone to bring about precipitation. Accordingly

[1] With Mr. E. J. Hanna an attempt was made to apply the method of Thannhauser and Setz (10) without success.

we undertook a detailed study of this point, the results of which are described in this paper.

Kirk (5) copied Bloor (3) in prescribing that the phospholipids should be precipitated from petroleum ether solution with *7 cc. of acetone and 3 drops of a saturated solution of magnesium chloride in 95 per cent alcohol.* However Boyd (11) for his lipid analyses on blood uses 7 cc. of acetone and *0.1 cc. of 30 per cent magnesium chloride in 95 per cent alcohol.* Accordingly both concentrations of magnesium chloride were employed.

In brief, this study has made it clear that the amount and concentration of the magnesium chloride solution used have a profound effect on the accuracy and the significance of the results obtained by methods that involve precipitation of the phospholipids by acetone. The percentage of ether-insoluble phospholipids is a function, more or less linear, of the amount of magnesium chloride added. However, the source of the phospholipid is also of great importance. All other conditions being constant, the percentage of ether-insoluble phospholipid may reach about 90 to 100 per cent of the total phospholipid of blood plasma and yet it rarely exceeds 20 per cent of the phospholipid of various tissues. The N:P ratios of the total phospholipid and of the ether-insoluble fraction indicate that the latter does not consist primarily of sphingomyelins but rather is merely a portion of the mixture of phospholipids present.

EXPERIMENTAL

Phosphorus was determined by the method of Fiske and Subbarow (12), perchloric acid (13) being used for digestion. A Klett-Summerson photoelectric colorimeter was used. Nitrogen was determined by the micro-Kjeldahl method. Choline was determined by the method of Brante (14), slightly modified.

All solvents were redistilled. Acetone was dried over drierite or anhydrous calcium chloride.

Experiments with Saturated Magnesium Chloride[2]

On Plasma Extracts—A suitable volume of heparinized plasma from a normal man and from a dog was extracted with about 20

[2] The saturated solution of magnesium chloride was prepared as follows: 10 cc. of alcohol were pipetted into a weighed glass-stoppered flask and

volumes of 3:1 alcohol-ether and filtered. The proteins were washed several times with small amounts of alcohol-ether and the filtrate made up to a suitable volume. An aliquot was taken for phosphorus determination. The remainder was evaporated to dryness at 40° under reduced pressure and in a continuous small stream of nitrogen. The residue was repeatedly extracted with petroleum ether and, in the case of human plasma, by chloroform as well. The centrifuged extract was made to volume. An aliquot was taken for phosphorus determination and then suitable aliquots were pipetted into a series of 15 cc. centrifuge tubes. The petroleum ether was evaporated to 1 cc. in a stream of nitrogen. If chloroform was also present, all solvent was evaporated and the residue was dissolved in 1 cc. of petroleum ether. To each tube 7 cc. of acetone were added and then 0, 1, 2, etc., drops of the saturated magnesium chloride solution. After being vigorously stirred, the tubes were stoppered and set away in the refrigerator for at least 2 hours and generally overnight.

The tubes were centrifuged. The acetone was poured off, and the tube rinsed with 3 cc. of cold acetone. Now, 5 cc. of freshly distilled ether, saturated with water, were added and the contents of the tube were thoroughly stirred with the ether. The tubes stood for at least 10 minutes, generally longer, and then were centrifuged. The clear ether solution was carefully aspirated into a 25 × 200 mm. test-tube for phosphorus determination. Another 3 cc. of moist ether were added to each centrifuge tube, and the stirring, standing, and centrifuging were repeated. The ether was added to the first lot.

The contents of the centrifuge tube, consisting of a clear drop of

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weighed. Enough $MgCl_2 \cdot 6H_2O$ was added to approximate but not make a saturated solution, and the flask again weighed. When the salt had completely dissolved, successive small amounts of $MgCl_2 \cdot 6H_2O$ were added, until a small excess remained undissolved. Thus, the weight of $MgCl_2 \cdot 6H_2O$, within less than 1 per cent, required to saturate 10 cc. of alcohol was determined. This method avoided the uncertainty of determining the concentration by evaporating and drying a portion of the saturated solution. At the prevailing room temperature, 10 cc. of alcohol dissolved 9.40 gm. of $MgCl_2 \cdot 6H_2O$. From the total weight and the specific gravity, it was found that 1 cc. of the saturated solution contained 596 mg. of $MgCl_2 \cdot 6H_2O$. Since the pipette used delivered 56 drops per cc., each drop carried 10.7 mg. of $MgCl_2 \cdot 6H_2O$.

water with a whitish layer on top, were transferred with several volumes of hot alcohol to a 25 × 200 mm. test-tube for combustion.

The results are shown in Figs. 1 and 2. The total acetone-insoluble phospholipid (A. I. P.) is the sum of the phosphorus in the ether-soluble and ether-insoluble fractions (E. I. P). Both the A. I. P. and the E. I. P. are expressed as a percentage of the total lipoid P, as determined on an aliquot of the petroleum ether extract. Thus values above 100 per cent are due to the summation of experimental errors.

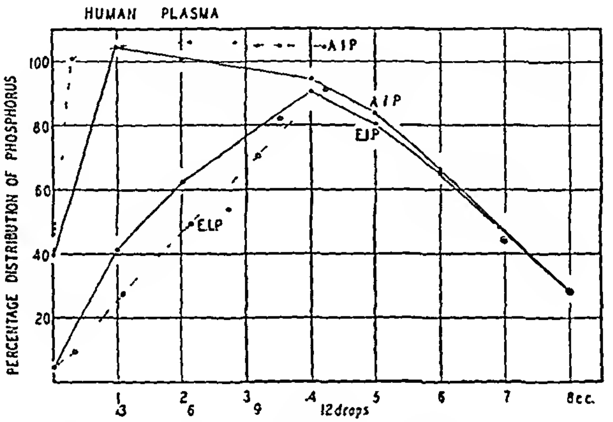

Fig. 1. Effect of amount and concentration of magnesium chloride solution on solubility of phospholipids in acetone and in moist ether. Experiment with human plasma. Each aliquot contained 0 0629 mg. of phosphorus. A. I. P , acetone-insoluble phospholipid; E I P., ether-insoluble phospholipid. Dash lines, saturated magnesium chloride; solid lines, 30 per cent magnesium chloride solution

On Phospholipids Isolated from Ox Serum and from Rat Tissues—Rat organs were ground with crushed glass and extracted by refluxing twice with 95 per cent alcohol and once with ethyl ether. The combined extracts were evaporated to dryness below 50° under reduced pressure. The residue was thoroughly extracted with ether. After removal of the suspended ether-insoluble material by centrifuging, the ether solution was concentrated in a stream of nitrogen and several volumes of acetone were added. No magnesium chloride was added. The acetone-insoluble fraction was redissolved in ether and reprecipitated with acetone.

This crude mixed phospholipid was then dissolved in ether and aliquots taken for weighing and analysis and for the precipitation experiments. The ethyl ether was evaporated and the residue taken up in 1 cc. of petroleum ether.

The nitrogen and phosphorus analyses are given in the legends to Figs. 3, 4, and 5 which show the yields of A. I. P. and of E. I. P. with increase in the amount of saturated magnesium chloride solution. It is quite obvious that the yields of E. I. P. from rat tissue phospholipids were much smaller than with human and dog plasmas. However, they were all alike in that the percentage of

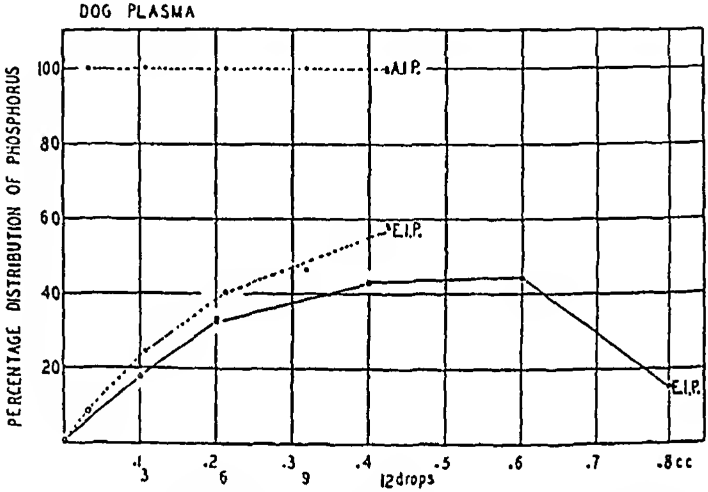

FIG. 2. Experiment with dog plasma. Each aliquot contained 0.0663 mg. of phosphorus. A. I. P., acetone-insoluble phospholipid; E. I. P., ether-insoluble phospholipid. Dash lines, saturated magnesium chloride; solid lines, 30 per cent magnesium chloride solution.

E. I. P. increased with increase in the amount of magnesium chloride.

It is interesting that the total acetone-insoluble lipid of brain was the only *tissue* preparation to give a measurable amount of E. I. P. in the absence of any magnesium chloride. And yet, with magnesium chloride, rat liver phospholipids gave practically the same yield of E. I. P. as did the acetone-insoluble lipids of the brain. Removal of the "protagon" that settled out of the brain lipids on standing in ether solution in the cold materially decreased the percentage of E. I. P. (*cf.* Figs. 4, *B* and 5).

Experiments were next carried out with phospholipid isolated

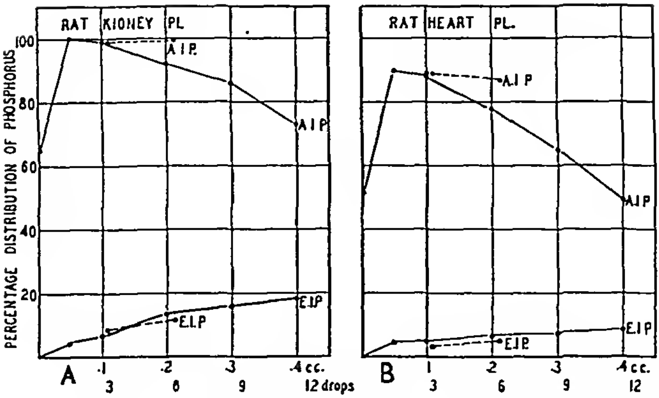

FIG. 3, *A*. Experiment with phospholipid isolated from rat kidneys. Analysis, N 3.11, P 3.38; N:P 2.04. Each aliquot contained 0.0639 mg. of phosphorus.

FIG. 3, *B*. Experiment with phospholipid isolated from rat hearts. Analysis, N 2.25, P 3.27; N:P 1.52. Each aliquot contained 0.0508 mg. of phosphorus. A. I. P., acetone-insoluble phospholipid; E. I. P., ether-insoluble phospholipid. Dash lines, saturated magnesium chloride; solid lines, 30 per cent magnesium chloride solution.

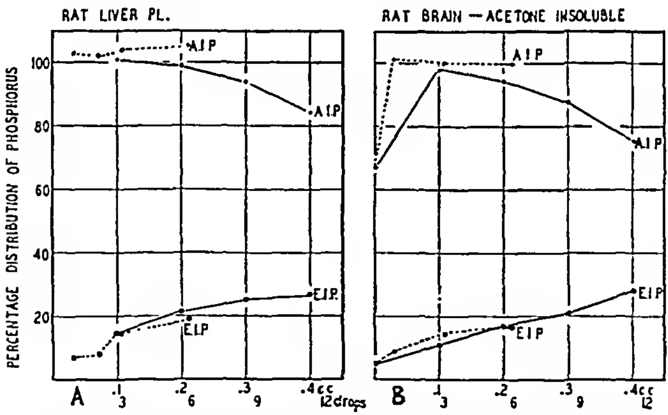

FIG. 4, *A*. Experiment with phospholipid isolated from rat livers. Analysis, N 2.45, P 3.08; N:P 1.76. Each aliquot contained 0.0500 mg. of phosphorus.

FIG. 4, *B*. Experiment with total acetone-insoluble lipids isolated from rat brains. Analysis, P 2.62. Each aliquot contained 0.0434 mg. of phosphorus. A. I. P., acetone-insoluble phospholipid; E. I. P., ether-insoluble phospholipid. Dash lines, saturated magnesium chloride; solid lines, 30 per cent magnesium chloride solution.

from ox serum, and thus still more comparable with that from tissues. Except for the extraction of the serum with 3:1 alcohol-ether, the method of isolation of serum phospholipids was the same as that used with tissues. Unpublished data show that the precipitation of serum phospholipids by acetone without magnesium chloride was less complete than in the case of tissue phospholipids.

The results of the experiments with ox serum phospholipid are shown in Figs. 6 and 7. It is quite clear that the behavior of the isolated phospholipid is essentially the same as in plasma extracts.

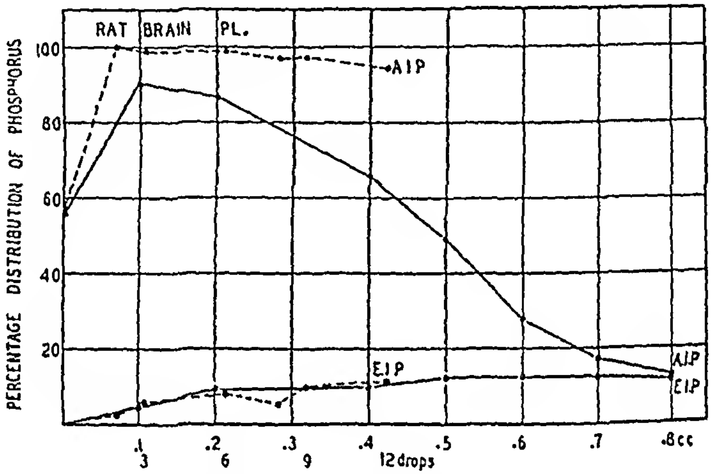

Fig. 5. Experiment with the ether-soluble fraction of the acetone-insoluble lipids isolated from rat brains. Analysis, P 3.28. Each aliquot contained 0.0527 mg. of phosphorus. A. I. P., acetone-insoluble phospholipid; E. I. P., ether-insoluble phospholipid. Dash lines, saturated magnesium chloride; solid lines, 30 per cent magnesium chloride solution.

Experiments with 30 Per Cent Magnesium Chloride Solution[3]

These experiments were carried out concurrently with those already described. The yields of A. I. P. and of E. I. P. are shown in Figs. 1 to 7.

These experiments with 30 per cent magnesium chloride show that the alcohol which is used as a vehicle tends to inhibit the precipitation of phospholipids.

In the two instances in which it was tried (Fig. 3), 0.05 cc. of 30 per cent magnesium chloride in 7 cc. of acetone produced maximum

[3] The 30 per cent solution was prepared by dissolving 15 gm. of $MgCl_2 \cdot 6H_2O$ in 95 per cent alcohol and bringing to 50 cc.

precipitation, although in one case it was only 90 per cent complete. In general it may be said that with volumes up to 0.2 cc.

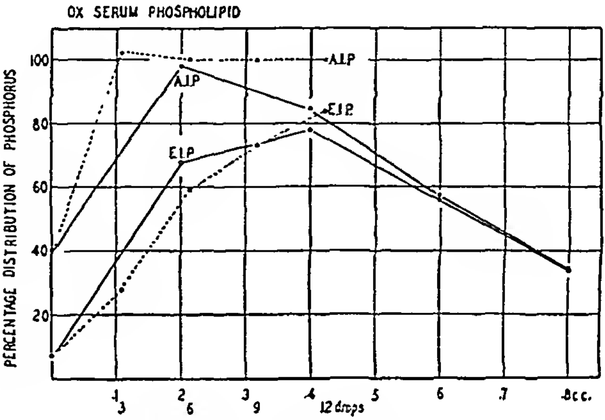

FIG. 6. Experiment with phospholipid isolated from ox serum. Analysis, N 2.80, P 1.90; N:P 3.27. Each aliquot contained 0.0501 mg. of phosphorus. A. I. P., acetone-insoluble phospholipid; E. I. P., ether-insoluble phospholipid. Dash lines, saturated magnesium chloride; solid lines, 30 per cent magnesium chloride solution.

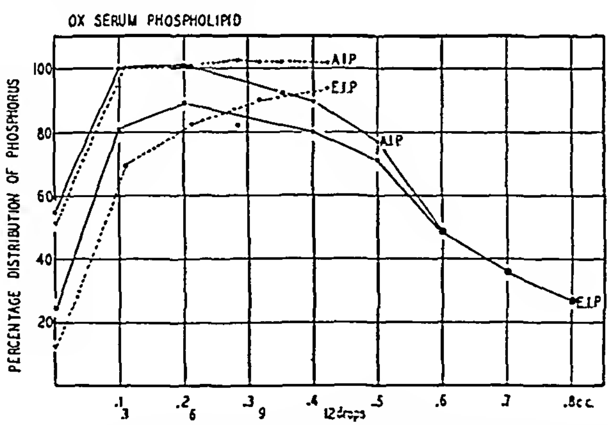

FIG. 7. Experiment with phospholipid isolated from ox serum. Analysis, N 2.57, P 2.25; N:P 2.53. Each aliquot contained 0.0366 mg. of phosphorus. A. I. P., acetone-insoluble phospholipid; E. I. P., ether-insoluble phospholipid. Dash lines, saturated magnesium chloride; solid lines, 30 per cent magnesium chloride solution.

the effect of the magnesium chloride is dominant; above 0.2 cc. the inhibitory effect of the alcohol becomes dominant. At the same time, the percentage of E. I. P., in that precipitated, steadily increases with the increase in amount of magnesium chloride added, even though the actual amount increases and then declines. Consequently, with about 0.6 cc. all of the phospholipid precipitated is insoluble in moist ether.

In the case of the tissue phospholipids, the yield of E. I. P. depends only on the amount of magnesium chloride added; the concentration, at least as between 30 per cent and saturated, has no effect. With plasma phospholipids, on the other hand, the differences in the percentage of E. I. P. with the same amount of magnesium chloride are quite considerable. There does not seem to be any explanation for these differences or their irregularity.

N : P Ratios of Plasma Extracts and of Ether-Insoluble Phospholipids

These experiments were carried out in order to determine whether or not the separation of phospholipids into the ether-soluble and ether-insoluble fractions actually produced a chemical fractionation.

As stated earlier, the determination of the N : P ratio on ordinary blood extracts is useless. However, Folch and Van Slyke (15) have introduced the method of precipitating the proteins and lipids with colloidal iron and magnesium sulfate, washing with water to remove non-lipid nitrogenous substances, and then extracting the lipids with alcohol and ether. The Folch-Van Slyke method was used on one sample each of dog and ox plasma. The alcohol-ether extract was evaporated to dryness at 40° in a stream of nitrogen and the residue was extracted with petroleum ether. Aliquots of this extract were taken for nitrogen, phosphorus, and choline analyses. Another aliquot, containing 0.675 mg. of lipoid P in the experiment on ox serum and 1.428 mg. of lipoid P in the experiment on dog plasma, was evaporated to 4 cc. and then 28 cc. of acetone and 48 drops of saturated magnesium chloride were added. The same procedure as that described above was followed, all volumes being multiplied by 4. The E. I. P. was also analyzed for nitrogen and phosphorus.

The results are given in Table I. The N : P ratios of the purified lipids show that, at the most, 47 per cent of the phospholipids of

the ox serum and 35 per cent of the phospholipids of the dog plasma consisted of sphingomyelins. And yet, on precipitation with acetone and magnesium chloride, 100 and 89 per cent, respectively, of the phospholipids were insoluble in moist ether. Clearly, the E. I. P. did not consist exclusively or mainly of sphingomyelins. Indeed, the N:P ratio of the E. I. P. was actually lower in both instances than the N:P ratio of the total phospholipids.

It should perhaps be pointed out that extraction by the Folch-Van Slyke method followed by solution in petroleum ether resulted

TABLE I

N:P Ratios of Plasma Extracts and of Ether-Insoluble Phospholipids

Method of extraction	Animal	Alcohol-ether-soluble P per 100 cc. plasma	Petroleum ether-soluble				Ether-insoluble fraction	
			P per 100 cc. plasma	Choline per 100 cc. plasma	N:P ratio	Choline:P ratio		N:P ratio
		mg.	*mg.*	*mg.*			*per cent*	
Ordinary alcohol-ether	Ox serum	3.74	3.48					
Folch-Van Slyke.	" "	3.22	2.81	8.8	1.47	0.80	101	1.37
Ordinary alcohol-ether.....	Dog plasma	14.01	13.18	45.2	3.98	0.88*	33	5.89
Folch-Van Slyke.	" "		11.91	37.2	1.35	0.80*	89	1.24

* It is likely that one or other of these ratios is in error. The decrease from 0.88 to 0.80 would mean an absolute as well as relative increase in the cephalin content.

in a significant decrease from the amount of phosphorus extracted in the usual way by alcohol-ether and then by petroleum ether.

SUMMARY

1. Under the standard conditions employed (0.3 to 2 mg. of phospholipid in 1 cc. of petroleum ether and 7 cc. of acetone), acetone alone precipitated about 40 to 70 per cent of the phospholipid of plasma and tissues. Addition of 1 drop of saturated or 0.1 cc. of 30 per cent $MgCl_2 \cdot 6H_2O$ in 95 per cent alcohol brought about complete precipitation. With more than 0.2 cc. of 30 per cent $MgCl_2 \cdot 6H_2O$, precipitation again became incomplete.

2. In the absence of $MgCl_2 \cdot 6H_2O$, all of the tissue phospholipid

and most of the plasma phospholipid that was precipitated were soluble in moist ether. With the addition of $MgCl_2 \cdot 6H_2O$, the percentage of ether-insoluble phospholipid increased in proportion to the amount of salt used. A maximum of about 20 per cent of the tissue phospholipid and about 90 per cent of the plasma phospholipid became insoluble in moist ether.

3. N:P ratios of plasma phospholipids and of the ether-insoluble fractions show that the latter do not consist solely or even mainly of sphingomyelins. The data indicate that the ether-insoluble fraction is simply a portion of the whole mixture of phospholipids.

4. These findings have an important bearing on the accuracy and significance of the results obtained with methods that involve precipitation of the phospholipids.

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STUDIES ON THE CHEMISTRY OF THE FATTY ACIDS

IX. A SPECTROSCOPIC STUDY OF METHYL ARACHIDONATE PURIFIED BY CRYSTALLIZATION AND DISTILLATION AND ITS ALKALI ISOMERIZATION PRODUCT

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In an earlier paper of this series (1) a comparison was made of the physical and analytical constants of samples of methyl arachidonate prepared by crystallization and distillation with those prepared by reduction of the octabromide. Low polybromide numbers of the latter preparations indicated some contamination by geometrical isomers, such impurities later being shown to exist in the case of linoleic and linolenic acids to the extent of 10 to 15 per cent of preparations made by debromination (2). The material prepared by crystallization followed by fractional distillation, however, was only 95 per cent pure (as indicated by the iodine number) and both samples exhibited diene numbers indicative of the presence of about 5 per cent of a conjugated acid, although rearrangement of methyl arachidonate to that extent under the conditions of the diene number determination was not precluded.

In order to establish the nature of the contaminating substances, a more critical study of the crystallization procedure and a spectroscopic examination of the products obtained is now reported. The alkali isomerization of arachidonic acid has also been followed spectroscopically, the results permitting certain observations pertinent to the structure of arachidonic acid. The conclusions with regard to this structure will be reported in Paper X of this series.

* Presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Gradute School of The Ohio State University.

EXPERIMENTAL

Preparation of Methyl Arachidonate—The source material used was the phosphatide fraction of the lipids of beef suprarenals.[1] Conversion of the fatty acids to methyl esters and preparation of methyl arachidonate by reduction of the octabromide were accomplished by essentially the same procedure as that reported by Shinowara and Brown (1). In the preparations made by purely physical means, their methods were also employed for the concentration of the methyl arachidonate in the filtrates obtained by crystallization at low temperature. The concentrate of methyl arachidonate was fractionally distilled at 1.0 to 2.0 mm. through a column 65 cm. long and 25 mm. inside diameter packed with single turn glass helices and equipped with a heating jacket controlled by a variable transformer. The column was connected with a multiple receiver enabling the fractions to be changed without disturbing the distillation and preventing air from entering the system. Mercury-sealed ground glass joints were used throughout and the system was thoroughly flushed out with an inert gas such as methane or nitrogen before commencement of the distillation. A reflux ratio of about 3:1 and a rate of distillation of about 60 gm. per hour were maintained. A longer column (100 cm.) of similar design was found to give a product of no greater purity and consequently was not further used, since the higher temperatures required in the distilling flask sharply increased the amount of polymerized residue. The desired product distilled at nearly constant temperature, usually in the vicinity of 163°, depending on the vacuum obtained. In this manner some 500 gm. of 94 to 95 per cent methyl arachidonate were prepared.

A graphical analysis of the course of the distillation is shown in Fig. 1. A linear relationship between refractive index and mole fractions (as measured by iodine number) is to be expected in a two component system where a nearly perfect solution is formed. The lower boiling fractions are found to lie on such a line which if extrapolated to iodine number 85.6 (theory for methyl oleate) will pass exactly through the known refractive index for methyl oleate; *i.e.*, 1.4522. The presence of oleic acid in large quantity in this source material has previously been reported (3).

[1] Courtesy of Oliver Kamm of Parke, Davis and Company.

Extrapolation of the line on which the low boiling fractions lie to iodine number 319.0 (theory for methyl arachidonate) gives the refractive index for 100 per cent methyl arachidonate. In addition to the lower boiling component, methyl oleate, there is a higher boiling component, fractions of which lie on a second line upon which extrapolation to iodine number 319.0 intersects the

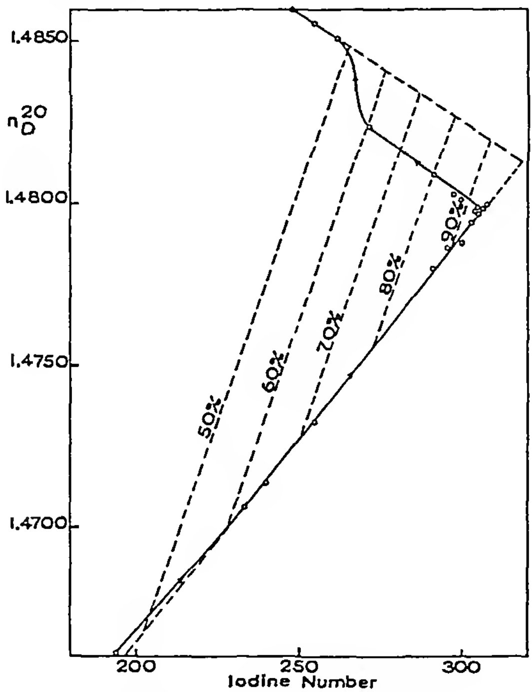

Fig. 1. Graphical analysis of the course of fractional distillations involved in the preparation of methyl arachidonate.

point representing the refractive index of pure methyl arachidonate. This higher boiling, viscous, yellow material was not isolated in a pure state, but determinations of its absorption spectrum, iodine number, molecular refraction, molecular weight, and carbon and hydrogen content indicated that it was mostly an oxidized (and possibly isomerized) form of methyl arachidonate. The assumption that at least part of this material has had only one double bond destroyed by oxidation and the arbitrary assign-

ment of iodine number 226.5 to the higher boiling substance permit the construction of isopercentile lines and the approximate evaluation of fractions lying inside the triangle defined by the three components.

A comparison of the physical and analytical constants of the 95 per cent product calculated to 100 per cent purity with those of the best samples prepared by debromination is given in Table I. The significance of these values will be discussed later.

TABLE I

Physical and Analytical Constants of Methyl Arachidonate Prepared by Different Methods

Constant	Reduction of bromide	Crystallization and distillation		Theory
			Calculated to 100 per cent purity	
Iodine No.	319.0*	308.0	319.0	319.0
n_D^{20}	1.4824*	1.4800	1.4813	
d_4^{20}	0.9098	0.9066	0.9082	
Mol. wt.	318.0*	317.0	318.1	318.5
Molecular refraction	99.88		99.76	99.05†
Polybromide No.	86.3*	88.1	92.7	
Thiocyanogen "	161.1*	159.0	163.1	159.5‡
Diene No.	7.9*	9.1*		
$E_{1\,cm.}^{1\%}$ at 235 mμ	12.9	17.3		
" " 270 "	3.5	2.5		
" " 300 "	1.7	0.1		

* From Shinowara and Brown (1).
† With the values of Swietoslowski (4).
‡ For two double bonds.

Alkali Isomerization of Arachidonic Acid—The alkali isomerization of arachidonic acid was studied to compare its behavior with that of linolenic acid which Kass and Burr (5) have isomerized to pseudoeleostearic acid (10,12,14-octadecatrienoic acid). The procedure employed was a modification of that used by them for linolenic acid isomerization. 20 gm. of 95 per cent methyl arachidonate were added to a like quantity of potassium hydroxide dissolved in 100 cc. of ethylene glycol which had been placed in an egg-shaped 3-necked flask heated to 150° in an oil bath maintained

at that temperature. A stream of nitrogen bubbling through the solution provided agitation and prevented atmospheric oxidation. At intervals, 10 cc. aliquots were removed, the acids were recovered, and the absorption spectrum measured in absolute alcohol solution. The instrument used was a Bausch and Lomb medium quartz spectrograph giving a linear dispersion from 210 to 700

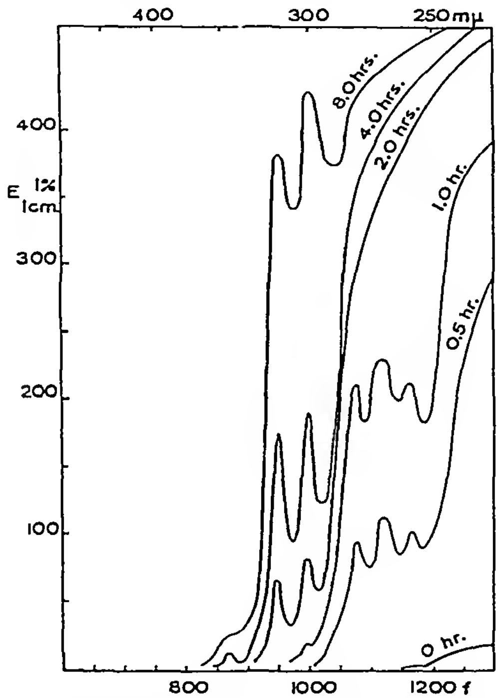

Fig. 2. Spectroscopic changes in the alkali isomerization of arachidonic acid in ethylene glycol at 150°.

mμ of 21 cm. This was equipped with a Hilger logarithmic sector photometer and details of the procedure used are given by Brode (6). A slit width of 0.10 mm. was employed except for the extinction values lower than 230 mμ, when it was increased to 0.30 mm.

Fig. 2 shows the changes in absorption spectra as the double bonds assumed a conjugated configuration. The extinction

values at 235, 270, and 300 mμ, representing the quantities of two, three, and four double bonds in conjugation respectively, are plotted against time in Fig. 3. It is noted that the rate of development (calculated from the initial slope) of the two and three bond system proceeds 7.1 and 5.1 times as fast respectively as that of the final four bond conjugated system.

The product after 8 hours saponification was acidified and crystallized from petroleum ether solution at −20°, yielding fine

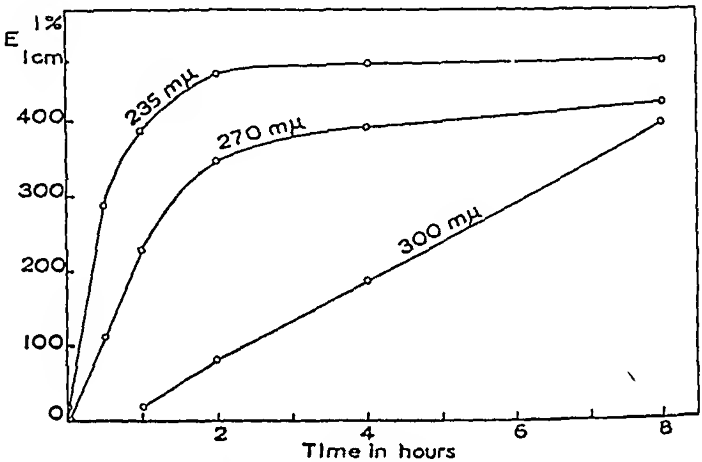

FIG. 3. Rates of formation of isomeric acids in the alkali isomerization of arachidonic acid.

white plates melting at 88–96°, the melting point being raised by recrystallization from 95 per cent alcohol to 95–98°.

$C_{20}H_{32}O_2$ (304.4). Calculated, C 78.9, H 10.6; found, C 78.74, H 10.48

Upon standing in contact with light and air the material oxidized and polymerized rapidly, becoming sticky and assuming a yellowish cast. After some time a portion, presumably a polymer, became relatively insoluble in hot alcohol.

The isomerized acid, upon recrystallization, exhibited three well defined absorption maxima in absolute alcohol at frequencies of 952, 1000, and 1048 *f* and an inflection at 1100 *f* with respective values of $E^{1\%}_{1\,\text{cm.}}$ of 2200, 2400, 1900, and 700. These bands correspond closely to the 20th, 21st, 22nd, and 23rd multiples of a fundamental infra-red band at 47.6 *f* and closely check the ab-

sorption spectra reported by Kuhn and Grundmann (7) for 2,4,6,8-decatetrene (f = fresnel = vibrations per second $\times 10^{12}$).

DISCUSSION

The inability to obtain methyl arachidonate of purity greater than 95 per cent by distillation would seem to indicate either that the maximum efficiency of the column had been reached or that a constant boiling mixture of 95 per cent methyl arachidonate and 5 per cent methyl oleate existed. The former explanation would seem to be discredited, since 95 per cent material has easily been obtained by Shinowara and Brown (1) by the use of a Claisen flask with a 25 cm. indented side arm and a 75 cm. vacuum-jacketed column as well as by the use of 65 cm. and 100 cm. packed columns in the present investigation. However, no final evidence for the existence of such an azeotropic mixture has been obtained.

The data in Table I do not in general show appreciable differences between the preparations made by reduction of the octabromide and those made by purely physical methods when contants for the latter are calculated to 100 per cent purity. Exaltation of the molecular refraction of the order of only 0.7 to 0.8 should not be considered significant in view of the high moleular weight of methyl arachidonate, and this calculation rather substantiates previously determined diene number determinations which show that the amount of conjugation present is very small. A more sensitive examination for conjugation was afforded by the absorption spectra determinations which showed that neither sample contained more that 0.5 to 1.0 per cent conjugation. The spectrum of the sample prepared by debromination showed small maxima at 270 and 300 mμ indicative of the presence of slight traces of impurities containing three and four double bonds in conjugation respectively. This material undoubtedly arises in the debromination process through contact with zinc bromide, since Turk (8) has shown that such compounds as magnesium chloride and zinc bromide are effective catalysts for the rearrangement of the double bonds of diolefins to a conjugated position.

The only significant difference between the products prepared by the two methods is in the polybromide number, acids obtained by debromination not being exactly characteristic in this respect of the naturally occurring acid (2). It is suggested that the cor-

rected value 91.8, which is the average of several samples prepared by physical methods, be substituted for the figure 86.5 in the formula of Ault and Brown (9) for the quantitative estimation of methyl arachidonate.

In Fig. 3 it is noted that the rate of formation of the three bond conjugated system (270 mμ) is comparable to that reported by Kass and Burr (5) for the isomerization of linolenic acid. It would seem not unlikely, then, that the same 1,4,7-triene system present in linolenic acid is also present for at least three of the double bonds in arachidonic acid.

SUMMARY

1. A critical study of the method of isolating methyl arachidonate by crystallization and distillation has been made and the product thus obtained compared with that prepared by reduction of the octabromide.

2. The absorption spectra of these samples show even better than the diene number and molecular refraction that these samples are relatively free from conjugated unsaturation.

3. A correction has been recommended for the empirical factor in the equation for the estimation of methyl arachidonate by the polybromide number.

4. The kinetics of the alkali isomerization of arachidonic acid have been followed spectroscopically and the similarity to the behavior of the 1,4,7-triene system present in linolenic acid noted.

5. The alkali isomerization product of arachidonic acid has been described.

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STUDIES ON THE CHEMISTRY OF THE FATTY ACIDS

X. THE STRUCTURE OF ARACHIDONIC ACID AS EVIDENCED BY OXIDATIVE DEGRADATION AND SELECTIVE HYDROGENATION

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Increasing interest in the structure of arachidonic acid has been aroused in recent years through the establishment (1, 2) of the fact that it is the most potent of the several unsaturated fatty acids reported to have a curative effect on the fat deficiency disease of rats. Some evidence has been offered by Nunn and Smedley-MacLean (3) that linoleic acid is the precursor of arachidonic acid, the latter being in the opinion of these authors the substance which is closely associated with the ability of the animal to store fat.

The degree and type of unsaturation of arachidonic acid have been well established by iodine number titrations, analyses of the octabromo and octahydroxy addition compounds, diene number determinations, and spectroscopic data (4–6). These data together with the demonstrated relationship of arachidonic acid to *n*-eicosanoic acid indicate a normal monobasic 20-carbon fatty acid possessing four double bonds, none of which is conjugated or closer to the carboxyl group than the 5-6 position, since the theoretical iodine number is easily obtained.

The present investigation is a continuation of the work initiated by Shinowara and Brown, who stated in a preliminary report (5) that the isolation of acetaldehyde, acetic acid, succinic acid, and a substance appearing to be adipic acid from degradations by ozonolysis and by oxidation with potassium perman-

* Presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Graduate School of The Ohio State University.

ganate in acetone solution indicated that the most probable structure was 6,10,14,18-eicosatetrenoic acid, a formula which they emphasized was only tentative. Several months before the completion of the present study, there appeared another preliminary communication on the structure of arachidonic acid by Dolby, Nunn, and Smedley-MacLean (7). Upon admittedly incomplete evidence based on an aqueous alkaline permanganate oxidation of arachidonic acid from which oxalic acid was definitely characterized and indications of the presence of glutaric, succinic, caproic, valeric, and formic acids were obtained, these authors suggested that the structure was 5,8,11,14-eicosatetrenoic acid, a formula which nicely accomodated their hypothesis mentioned above that linoleic acid is the precursor of arachidonic acid in the animal body.

In the present paper are reported the results of three degradations of methyl arachidonate, two by ozonolysis and one by oxidation with potassium permanganate in acetone solution, together with a study of the selective hydrogenation of methyl arachidonate. The principal difficulty of the previous investigations (5, 7) seemed to lie in the fact that insufficient quantities of degradation products were obtained for definite isolation and characterization, a situation arising either from low yields or an insufficient quantity of starting material. In the present work, relatively large samples were used in each degradative experiment and certain modifications of the usual procedures were employed to insure better yields. In the ozonolysis experiments, the ozonization was conducted at dry ice temperatures in order to minimize if possible the attack upon the double bonds by molecular oxygen, an undesirable reaction which results in unhydrolyzable peroxides and polymers. A method of oxidative hydrolysis of the ozonide with hydrogen peroxide was also employed to simplify the types of products obtained and eliminate aldehyde polymerization. The acids were characterized by boiling point or melting point, neutral equivalent, and melting point and mixed melting point of at least two derivatives of which the amide and phenacyl, *p*-bromophenacyl, and *p*-nitrobenzyl esters were commonly used. When authentic samples of derivatives were not available for comparison by mixed melting point, the identity of the acid was confirmed by ultimate analysis of the acid and one derivative.

In Table I are given the quantities of products isolated from the three degradations of methyl arachidonate and their percentage yields calculated on a molar basis. In the first ozonolysis and acetone-permanganate degradations, samples of the 95 per cent methyl arachidonate prepared by crystallization and distillation as described in the preceding report of this series (6) were used as the starting material. In the second ozonolysis experiment a sample prepared by reduction of the octabromide was employed.

TABLE I

Products of Oxidative Degradation of Methyl Arachidonate

Product	Ozonolysis I, 110 0 gm.		Ozonolysis II, 48.0 gm.		Acetone-$KMnO_4$, 39.0 gm.	
	gm.	*per cent yield*	*gm.*	*per cent yield*	*gm.*	*per cent yield*
Malonic acid			1.2	8		
Carbon dioxide	*	>200	10.5	157		
Acetaldehyde	Qualitatively only		4.9†	74		
Acetic acid	10.4	49	4.5	50		
Oxalic "					5.3	39
Caproic "	9.5	24	5.4	31	3.8	28
Glutaric "	18.8	41	4.4‡	22	7.5	49
Succinic "	4.6	11	6.3§	35	5.1	38

* Estimated from rate of evolution.

† As sodium bisulfite addition compound.

‡ Includes 1.7 gm. of probable monomethyl glutarate.

§ Includes 1.6 gm. of probable monomethyl succinate.

Under ordinary conditions of ozonide hydrolysis in boiling water malonic acid and its semialdehyde decompose to give carbon dioxide, acetic acid, and acetaldehyde, while under the conditions of the acetone-permanganate degradations, oxalic acid is formed as a secondary decomposition product. Inasmuch as glutaric acid, and to a lesser extent succinic acid, was found to yield carbon dioxide when treated with 5 per cent hydrogen peroxide in boiling water, the determination of carbon dioxide evolution was made prior to the introduction of the oxidizing agent into the hydrolysis flask, so that it would be a valid index of the number of $=CH-CH_2-CH=$ fragments present in the original molecule.

In the first ozonolysis experiment the yield of carbon dioxide was estimated from the rate of evolution of comparable molar quantities of malonic acid in boiling water and the amount of acetaldehyde was not quantitatively determined. In the second ozonolysis experiment preliminary hydrolysis was carried out for 36 hours below the decomposition temperature of malonic acid solutions and the liberated malonic acid was recovered. Only partial hydrolysis was expected in this short a time, since Erdmann, Bedford, and Raspe (8) reported only 30 per cent yields of malonic acid from linolenic acid ozonide after 9 days of similar hydrolysis. The remaining unattacked ozonide was then decomposed in the usual fashion, the total quantity of carbon dioxide evolved being determined instead of estimated as before. It was felt that the relatively low yields in the first experiment may have been due in part to incomplete oxidation of the aldehydes so a much more vigorous hydrogen peroxide treatment was given the net result, however, being an increase in the ratio of succinic to glutaric acid found. In this connection it might be pointed out that in the blank determinations of the stability of glutaric acid to the conditions of hydrogen peroxide hydrolysis mentioned above, succinic acid was readily identified as a decomposition product. Its isolation in the experiments on methyl arachidonate therefore, was not necessarily an indication of the presence of the $=CH-(CH_2)_2-CH=$ fragment in the original molecule Since both the $=CH-(CH_2)_2-COOCH_3$ and $=CH-(CH_2)_3-COOCH_3$ groups could not have been present, the view that the succinic acid arose wholly from secondary decomposition of glutaric acid was somewhat substantiated by indications of both monomethyl succinate and monomethyl glutarate, although definite proof of the presence of these substances was lacking.

The results of these three degradations led to the following conclusions regarding the structure of arachidonic acid, (1) the presence of the $=CH-(CH_2)_4-CH_3$ fragment placing the terminal double bond in the 14-15 position as evidenced by the isolation of caproic acid, (2) the presence of two or three $=CH-CH_2-CH=$ fragments as evidenced by the isolation of malonic acid and its secondary decomposition products acetaldehyde, acetic acid, oxalic acid, and carbon dioxide, (3) the presence of the $=CH-(CH_2)_3-CH=$ or the $=CH-(CH_2)_3-COOH$ fragment

as evidenced by the isolation of glutaric acid. In view of the facts that the iodine number would be low if the first double bond were closer to the carboxyl than the 5-6 position (9) and that the acid contains no conjugated unsaturation (6), the only possible configuration to assign for arachidonic acid is 5,8,11,14-eicosatetrenoic acid, a confirmation of the proposal of Dolby, Nunn, and Smedley-MacLean (7). We prefer to regard the succinic acid as arising from secondary decomposition of the glutaric acid, although its derivation in part from a small quantity of an isomeric ester is not precluded.

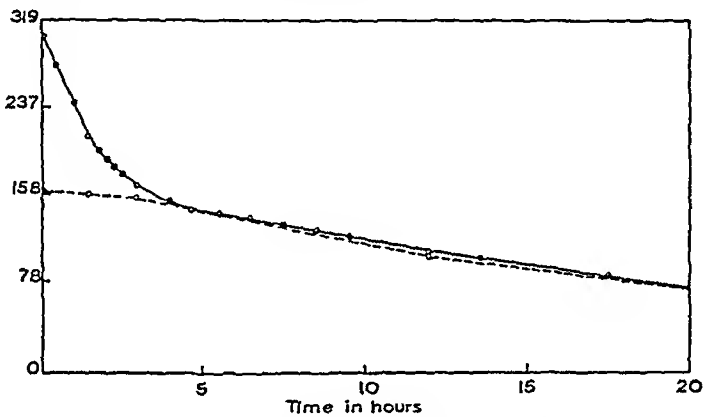

FIG. 1. Hydrogenation of methyl arachidonate at 82° with nickel-on-kieselguhr catalyst. Solid line, ○ iodine No. found, ◒ iodine No. calculated from hydrogen absorption; broken line, ○ thiocyanogen No. found.

In order to obtain further confirmation of this assignment of structure a study of the catalytic hydrogenation of methyl arachidonate was undertaken. When platinum black was used as a catalyst, 4 moles of hydrogen were rapidly absorbed at room temperature and atmospheric pressure with no indication of selectivity of action. With a catalyst of nickel supported on kieselguhr at 3 atmospheres pressure at 70–80° absorption took place in two distinct stages, as noted in Fig. 1, two of the double bonds apparently reacting some 15 times faster than the other two double bonds. The measured absorption of hydrogen was found to check within 1 to 2 per cent with the iodine number of the product.

Ozonolysis of 24.0 gm. of the intermediate diethylenic reduction product which had been purified somewhat by crystallization at low temperature gave the yields indicated in Table II. If the succinic acid arose from secondary decomposition of glutaric acid by hydrogen peroxide, the ratio of the quantity of these two acids to the amount of suberic acid indicated that the diethylenic acid was composed of 80 to 90 per cent 5,14-eicosadienoic acid and 5 to 10 per cent 8,14-eicosadienoic acid. The presence of small amounts of a contaminating trienoic acid was indicated by the traces of malonic acid secondary decomposition products and was expected from the iodine number of the starting material

TABLE II

Yields on Ozonolysis of 24 Gm. of Intermediate Diethylenic Reduction Product

Product	Weight	Yield
	gm.	*per cent*
Acetaldehyde	Amount very small	
Acetic acid	0 33	7
Carbon dioxide	0 57	17
Caproic acid	4 4	51
Succinic "	0 7	8
Glutaric "	3 5	36
Adipic acid	1.5	14
Suberic "	0.4	3
Azelaic "	1.7	12

which was slightly higher than the theory for methyl eicosadienoate. This indication that the double bonds most reactive to hydrogen are the central ones paralleled the results of van der Veen (10) whose studies on linolenic acid showed that the 12-13 or middle double bond was reduced most rapidly. More significant, of course, was the confirmation afforded the above assignment of structure to arachidonic acid. In this respect the identification of adipic, suberic, and azelaic acids further substantiated the identity and relative positions of the fragments indicated by degradation of the unreduced ester.

Preliminary, and as yet incomplete, experiments on the thiocyanogen numbers of hydrocarbon dienes indicated that under customary analytical conditions (11) thiocyanogen attacks

1,4-, 1,5-, and 1,6-diene systems at approximately 1, $1\frac{2}{3}$, and $1\frac{5}{6}$ double bonds respectively. This would lead to the prediction that the thiocyanogen number of methyl arachidonate should undergo no appreciable change in the absorption of the first 2 moles of hydrogen. This was found to be substantially true, as is seen in Fig. 1.

EXPERIMENTAL

Ozonolysis I—The ozonizer used[1] for this investigation has been described in detail by Wiest (12). A total of 110.0 gm. of 95 per cent methyl arachidonate prepared by crystallization and distillation (6) in 500 cc. of methylene chloride was ozonized at dry ice temperatures and hydrolyzed in four batches to prevent excessive loss in case of explosion. The general procedure for the hydrolysis of the ozonide was as follows: The ozonide solution and about 3 times its volume of water were placed in a 3-necked flask equipped with a mercury-sealed stirrer, an inlet tube for a slow stream of carbon dioxide-free air, a dropping funnel, and a reflux condenser leading in succession to two dry ice-cooled traps to condense the solvent (b.p. 41°) and any volatile decomposition products, a sulfuric acid bubbler, drierite tube, ascarite absorption tube, and a drierite-ascarite guard tube. While vigorous stirring was maintained, the methylene chloride was removed by cautiously warming the suspension. Then vigorous boiling of the aqueous suspension was maintained for 2 or more hours while the evolution of carbon dioxide was measured. A 50 per cent excess of a 30 per cent solution of hydrogen peroxide was slowly introduced through the dropping funnel and boiling continued for an hour longer. The solution was then steam-distilled and the distillate made alkaline, extracted with ether, and evaporated to dryness. In this fashion the ozonide degradation products were divided into four main fractions, carbon dioxide as measured in the absorption tube, volatile products in the dry ice traps, steam distillate, and steam distillation residue.

As mentioned earlier, the quantity of carbon dioxide evolved in this experiment was estimated from the rate of evolution. 0.1 mole of malonic acid was found to evolve carbon dioxide at an

[1] Courtesy of Professor A. L. Henne.

initial rate of 0.463 gm. per hour, while like quantities of the ozonide evolved 0.880 to 1.200 gm. per hour, indicating the presence of two to three malonic acid fragments resulting from degradation of 1 molecule of the ester.

The methylene chloride in the dry ice traps was washed successively with small portions of water, the aqueous solutions being neutral to litmus but giving positive tests with Schiff's reagent and the fuchsin-aldehyde reagent. Positive results were given in a color test specific for acetaldehyde. In this test a deep blue color is developed when the aqueous solution is treated with sodium nitroprusside and a secondary amine, in this case dibutylamine. The other normal aldehydes through caproaldehyde were found to give negative results. The 2,4-dinitrophenylhydrazone melted at 168° and was not depressed by an authentic sample, m.p. 168°. These data provided qualitative identification of acetaldehyde.

The dry sodium salts of the steam distillate acids were placed in 85 per cent phosphoric acid and the free acids upon distillation formed two immiscible layers. These layers were redistilled separately, the upper layer acid boiling at 198–204°, neutral equivalent 114.3, amide m.p. 99–100°, *p*-bromophenacyl ester m.p. 71.5–72.0°. The corresponding literature values for caproic acid are b.p. 205°, neutral equivalent 116.2, amide m.p. 101°, *p*-bromophenacyl ester m.p. 72°. No depressions of the melting points were observed when the samples were mixed. The lower aqueous layer was exhaustively extracted with ether, dried, the ether removed, and the residual acid was found to boil at 105–120°. The melting point of the *p*-bromophenacyl ester, 84.5–84.9°, was not depressed by an authentic sample of *p*-bromophenacyl acetate, m.p. 85°.

The steam distillation residue was evaporated to dryness, esterified with methyl alcohol, and distilled through a short column at 2 mm. pressure, b.p. 147–170°. Hydrolysis of the ester gave a mixture of dibasic acids of neutral equivalent 65.5 (theory for glutaric acid 66.0, for succinic acid 59.0). The *p*-bromophenacyl esters were easily separated into two fractions, one melting at 210–211° the other at 137°, the former being insoluble in hot alcohol. These melting points were not depressed by mixture with authentic samples of the succinate, m.p. 211°, and the glu-

tarate, m.p. 137°, respectively. The *p*-nitrobenzyl ester, m.p. 68–69°, was not depressed by the known glutarate, m.p. 69°, nor was the phenacyl ester, m.p. 103°, depressed by an authentic sample of the glutarate, m.p. 104°. Knowledge of the identity of the acids in the mixture then permitted easy separation by virtue of the low solubility of succinic acid in chloroform and the acids were found to melt at 93–96° (glutaric acid 99°) and 182–184° (succinic acid 185°). From the neutral equivalent of the mixture and the relative quantities of the two fractions obtained in the separation of the acids it was indicated that the ratio of glutaric to succinic acid was of the order of 4:1.

Ozonolysis II—48 gm. of methyl arachidonate prepared by debromination of the octabromide (6) were ozonized as previously described. In this case the ozonide was hydrolyzed at 40–50° for 36 hours during which time no appreciable evolution of carbon dioxide or acetaldehyde was detected. After addition of 60 cc. of 30 per cent hydrogen peroxide the suspension was extracted with ether.

The aqueous residue upon evaporation under a vacuum yielded 3.2 gm. of a crude oily residue from which 1.2 gm. of a crystalline acid were obtained, m.p. 128–130°, undepressed by a sample of malonic acid, m.p. 130–131°.

$C_3H_4O_4$.	Calculated.	C 34.6,	H 3.86,	neutral equivalent 52.0
	Found.	" 34.95,	" 4.01,	" " 52.6

When heated above its melting point at 135–140°, the acid decomposed, leaving no residue. When it was boiled with acetic anhydride, an amber-colored solution developed which had a pronounced green fluorescence. Oxalic, succinic, glutaric, and adipic acids gave negative results in this sensitive test for malonic acid.

The ether extract containing the bulk of the undecomposed ozonide was freed of solvent and rehydrolyzed in boiling water until the evolution of carbon dioxide had virtually ceased. Hydrogen peroxide was then added dropwise until the aqueous suspension gave a negative test with Schiff's aldehyde reagent, a process which required a large excess of the oxidizing agent (200 cc. of 30 per cent solution).

The acetaldehyde from the dry ice traps was precipitated as the bisulfite addition compound and weighed as such. Regenera-

tion of portions of aldehyde with hydrochloric acid enabled repetition of the qualitative tests mentioned earlier.

The presence of caproic and acetic acids in the steam distillate was confirmed by essentially the same methods as previously described.

Before evaporation of the steam distillation residue it was extracted with an equal quantity of ether. The ether-soluble fraction was distilled at 5 mm., yielding a small quantity of a colorless liquid which solidified when cooled at 0°. Preparation of the *p*-bromophenacyl esters yielded about equal quantities of the di-*p*-bromophenacyl succinate and glutarate. Since the boiling point and melting point of this ether-soluble fraction were far too low for a mixture of free succinic and glutaric acids, it was thought probable that this fraction consisted largely of a mixture of the monomethyl esters. Unsymmetrical methyl *p*-bromophenacyl esters of glutaric and succinic acids had been prepared and found to melt at 46.8° and 104.8° respectively (13) but the monomethyl esters of the acids could not be isolated from the degradation products in the form of these derivatives.

From the water-soluble portion of the steam distillation residue were obtained succinic and glutaric acids which were isolated and identified as previously described.

Acetone-Permanganate Degradation—47 gm. of the 95 per cent methyl arachidonate prepared by crystallization and distillation were oxidized in acetone by potassium permanganate by a modification of the method of Armstrong and Hilditch (14). The sample was placed in 2 liters of acetone and 500 gm. of finely powdered potassium permanganate were added in small portions, the mixture being refluxed after each addition and subsequently for 18 hours. The acetone was then removed from the precipitated manganese dioxide and potassium salts by filtration and the cake allowed to dry. The acetone solution was freed of solvent, neutralized with dilute sodium hydroxide, and extracted with ether. The ether extract contained 8.0 gm. of neutral unchanged ester. The acetone-free cake was washed exhaustively with dilute sodium hydroxide, a little sodium bisulfite being included in the first washings to reduce any unchanged potassium permanganate. The washings were combined with the aqueous alkaline solution, concentrated, acidified with hydrochloric acid, and steam-distilled.

The steam distillate was extracted with ether and the caproic acid obtained was characterized by boiling point, neutral equivalent, and *p*-bromophenacyl ester as previously described.

The aqueous solution of the steam distillation residue was purified somewhat by treatments with charcoal, evaporated to dryness, powdered in a mortar, and exhaustively extracted with chloroform from a Soxhlet apparatus. The crude extract had a neutral equivalent of 84.4 and readily yielded the *p*-bromophenacyl ester characteristic of glutaric acid. The residue was then thoroughly extracted with ether, yielding a crude extract of neutral equivalent 67.0 which was found to be a mixture of succinic and oxalic acids. The percentage of oxalic acid was determined by precipitation of calcium oxalate and also by direct titration with standard permanganate solution. Since the formation of *p*-bromophenacyl oxalate is an extremely slow reaction, the succinate was easily prepared from the mixture of acids as well as from a portion from which oxalic acid had been removed as the insoluble calcium salt.

Selective Hydrogenation—Considerable experimentation with catalysts, temperatures, and pressures of hydrogenation determined the conditions which are given below. In a Burgess-Parr laboratory hydrogenator at 82° in 100 cc. of absolute alcohol with 0.6 gm. of nickel-on-kieselguhr catalyst (40 per cent nickel), 10.14 gm. of methyl arachidonate absorbed hydrogen, as shown in Fig. 1. The slope of the curve was quite reproducible and permitted identical runs to be stopped after various intervals and analyzed for iodine and thiocyanogen numbers. The iodine number found usually checked the observed absorption of hydrogen within 1 to 2 per cent. A sample of 70.5 gm. of 95 per cent methyl arachidonate was reduced just short of the diethylenic stage and the product purified by crystallization from a 5 per cent solution in methyl alcohol. The four fractions obtained had the following iodine numbers.

−20° ppt.	2.5 gm.	2.8 iodine No.
−40° "	8.0 "	62.0 " "
−75° "	29.5 "	159.5 " "
−75° filtrate	28.0 "	210.6 " "

24 gm. of the −75° precipitate, iodine number 159.5 (theory for methyl eicosadienoate, 157.5), were ozonized and hydrolyzed as

previously described. Only 0.567 gm. of carbon dioxide was evolved. The dry ice trap contents gave the specific blue color test for acetaldehyde but the amount was so small that only a trace of the 2,4-dinitrophenylhydrazone was obtained. Steam distillation yielded caproic acid, which was characterized as before, but only a small amount of acetic acid was observed by titration of the aqueous portion.

The steam distillation residue was extracted with ether and from this fraction were recovered two acids, one soluble in chloroform, m.p. 95–99°, which proved to be azelaic acid.

$C_9H_{16}O_4$.	Calculated.	C 57.5,	H 8.58,	neutral equivalent 94.1
	Found.	" 57.14,	" 8.40,	" " 95.7

The *p*-bromophenacyl ester melted at 130.2–130.6° (literature, 131°).

$C_{25}H_{26}O_6Br_2$. Calculated, C 51.6, H 4.51; found, C 51.34, H 4.42

The other acid, insoluble in chloroform, m.p. 134–139°, proved to be suberic acid.

$C_8H_{14}O_4$.	Calculated.	C 55.2,	H 8.12,	neutral equivalent 87.0
	Found.	" 55.05,	" 7.98,	" " 86.7

The *p*-bromophenacyl ester melted at 143.0–143.5° (literature, 144°).

$C_{24}H_{24}O_6Br_2$. Calculated, C 50.8, H 4.26; found, C 50.64, H 4.23

The water-soluble portion of the steam distillation residue contained three acids; the one soluble in chloroform was found to be glutaric acid, m.p. 93–96°, neutral equivalent 67.2, *p*-bromophenacyl ester, m.p. 135–136°, which was not depressed by an authentic sample. The chloroform-insoluble fraction was found to be a mixture of adipic and succinic acids; the *p*-bromophenacyl esters melting at 151–152° and 209–210° were easily separated by crystallization from alcohol, the latter being insoluble in the hot solvent. The melting points were not depressed by mixing with authentic samples of the adipate, m.p. 154°, and the succinate, m.p. 211°, respectively. From the neutral equivalent of the mixture, 68.3, the ratio of succinate to adipate was estimated to be about 1:2.

SUMMARY

1. Ozonolysis of methyl arachidonate has been found to yield caproic, acetic, glutaric, succinic, and malonic acids, acetaldehyde, and carbon dioxide.

2. Oxidation of methyl arachidonate by potassium permanganate in acetone has been found to yield caproic, glutaric, succinic, and oxalic acids.

3. The structure of arachidonic acid has been assigned as 5,8,11,14-eicosatetrenoic acid.

4. The hydrogenation of methyl arachidonate has been found to proceed in two stages, the diethylenic intermediate product consisting of 80 to 90 per cent methyl 5,14-eicosadienoate and 5 to 10 per cent methyl 8,14-eicosadienoate.

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THE EFFECT OF CONDITIONS OF HYDROLYSIS AND OF PROLONGED HEATING UPON THE OPTICAL ROTATION OF SULFURIC ACID HYDROLYSATES OF ZEIN*

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Some time ago we observed that a sulfuric acid hydrolysate of zein prepared in an autoclave at 165° did not support growth in young rats when it was supplemented with certain amino acids, even though the same amino acids, added to a hydrolysate prepared by refluxing, permitted moderate growth. Since the optical rotation of the autoclaved hydrolysate was lower than that of the refluxed, either destruction or racemization of essential constituents, or both, might have occurred. Loss of amino nitrogen and production of extra ammonia upon prolonged autoclaving of proteins with acids have been noted by Van Slyke (1912) and others, but simultaneous observations on optical rotation have apparently never been made. To determine whether racemization also takes place, hydrolysates of zein prepared under conditions differing widely with respect to sulfuric acid concentration, temperature, and time were examined for optical rotation, amino nitrogen content, and in some cases for ammonia content.

EXPERIMENTAL

Zein was ground to pass a 40 mesh sieve; it gave a clear, but faintly pigmented solution in 75 per cent alcohol. On the air-dried basis it contained 4.86 per cent of moisture, 0.22 per cent of ash, and 1.29 per cent of ether-extractable material. On an ash- and moisture-free basis the nitrogen content was 15.53 per

* A preliminary report of the data in this paper was presented before the American Society of Biological Chemists at New Orleans, March, 1940 (Borchers and Berg, 1940).

cent; Chittenden and Osborne (1892) found 16.12, and Hoffman and Gortner (1925), 15.33 per cent.

The sulfuric acid solutions (aqueous) were approximately 4, 8, 14, 20, 25, and 33 per cent by volume; normalities by titration were 1.6, 3.1, 5.3, 7.5, 9.5, and 12.6. 10 cc. of acid solution were used in each test together with 1.856 gm. of zein, mixed intimately with 1.86 gm. of pumice and 0.48 gm. of acid-washed charcoal to increase the porosity of the doughy mass formed upon heating and to clarify the hydrolysate. The heating was conducted in an oil-jacketed thermoregulated autoclave at temperatures ranging from 120–200°, as well as under a reflux, for 1 to 60 hours. The autoclave was preheated to 100°; the recorded time of heating included the period of temperature rise above that point (about 1 hour for each 80°), but not the period of cooling.

The hydrolysates were filtered by suction while hot. Polarimetric readings were made at room temperature in a Schmidt-Haensch polariscope with a 1 dm. tube and a sodium vapor lamp. Correction for variations in temperature was found unnecessary between 20–40° (note also Winnick and Greenberg (1941)). Amino nitrogen was estimated according to Van Slyke, ammonia by titration of the distillate from an aliquot of the hydrolysate made alkaline with magnesium oxide, and total nitrogen (as a basis for comparisons) by the Kjeldahl method.

Fig. 1 presents the data on optical rotation and amino nitrogen obtained on the several hydrolysates prepared with 20 per cent sulfuric acid. Complete hydrolysis was marked by values of 2.5° ± 0.1°, 71 ± 1 per cent, and 20 ± 1 per cent for optical rotation and amino and ammonia nitrogen, respectively. Calculated on the same basis, the data of Hoffman and Gortner (1925) gave 70.1 and 18.06 per cent for amino and ammonia nitrogen. For ammonia nitrogen Osborne and Harris (1903) report 18.41, and Gortner and Blish (1915) 20.75 per cent.

In all instances changes in rotation approximately paralleled changes in amino nitrogen until maximum values for both were attained. Refluxing for 60 hours (36 hours beyond completion of hydrolysis) caused no appreciable decrease from either maximum. The observations on hydrolysis under a reflux are consistent with data obtained on casein during 33 hours of refluxing with hydrochloric acid (Winnick and Greenberg, 1941). A period several

days longer would probably have induced some change (Gortner and Holm, 1917). Heating in the autoclave much beyond the time required for complete hydrolysis caused decreases from

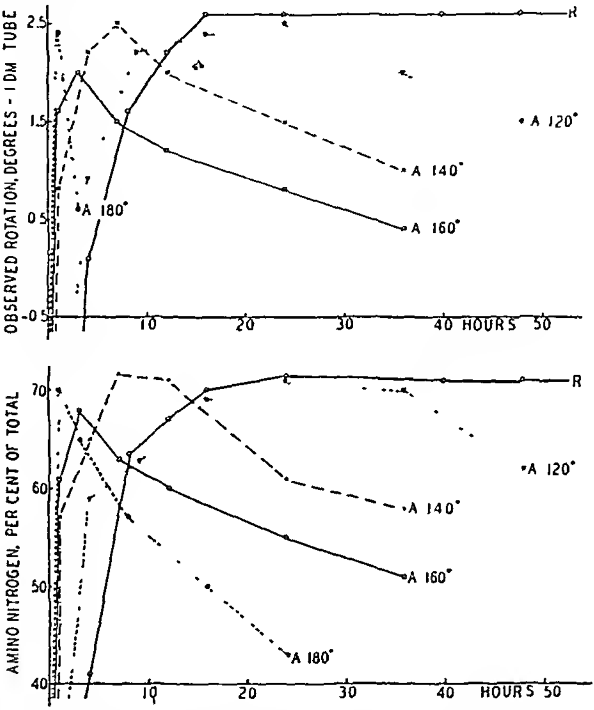

Fig. 1. Changes in optical rotation and amino nitrogen content upon heating zein with 20 per cent aqueous sulfuric acid solution. The curves connect points representing analytical results obtained on hydrolysates prepared by boiling under the reflux (*R*), or by heating in the autoclave (*A*) at the temperature specified, for various periods of time. "Observed rotations" are based on concentrations of 27.5 mg. of N per cc. The 1 hour hydrolysates prepared under a reflux and in the autoclave at 120° showed −5.3° and −3.6°; both yielded 29 per cent of amino nitrogen. Autoclaving at 200° for 2, 5, and 15 hours yielded 68, 58, and 50 per cent of amino nitrogen; the hydrolysates were too deeply pigmented for polarimetric observation, as were also those autoclaved at 180° for more than 3 hours.

both maxima. Increases in ammonia content accounted largely, though apparently not completely, for the decreases in amino nitrogen. Measured in relation to maximal values, the decreases

in rotation were proportionately greater than the decreases in amino nitrogen content. This divergence can logically be attributed to racemization.

The periods required for complete hydrolysis with 14, 25, and 33 per cent sulfuric acid under a reflux were approximately 30, 12, and 5 hours, respectively; in the autoclave at 120°, about the same; at 140°, 8, 6, and 3 hours; at 160°, 4, 2, and 1 hour. At 180° hydrolysis was complete in 1 hour with all concentrations of acid. In no instance did appreciable racemization or destruction occur in the 36 to 60 hours under the reflux or before completion of hydrolysis in the autoclave; continued autoclaving beyond that point induced both. The proportionate decreases in amino nitrogen and in optical rotation and the divergence between them were more marked at the higher temperatures; increase in acid concentration apparently had less influence than increase in temperature. Maximum amino nitrogen content and maximum optical rotation were never attained with concentrations of 4 and 8 per cent sulfuric acid; on prolonged heating at elevated temperatures excessive ammonia production occurred.

SUMMARY

During the course of the hydrolysis of zein with aqueous sulfuric acid, 14 to 33 per cent by volume, either under a reflux or in the autoclave at 120–180°, no appreciable racemization or destruction of amino acids occurred. Prolonging the refluxing to 36 to 60 hours had little or no effect, but autoclaving longer than necessary for hydrolysis induced both racemization and destruction, more markedly so at the higher temperatures. Concentrations of sulfuric acid as low as 8 per cent by volume are apparently not suitable for the complete and uncomplicated hydrolysis of zein.

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THREONINE DEFICIENCY IN HYDROLYSATES OF ZEIN PREPARED BY AUTOCLAVING*

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In a previous paper (Borchers and Berg, 1942) we showed that autoclaving zein with sulfuric acid longer than necessary for complete hydrolysis causes destruction and racemization; either of these might account for the failure of such a hydrolysate to promote growth in young rats when substituted for a hydrolysate, prepared by refluxing, in a diet which produced moderate growth. It seemed to us that this deterioration in the dietary protein might well be the result primarily of essential amino acid deficiencies which could be detected and overcome by appropriate supplementation. Because threonine is known to be present in zein in relatively small amount[1] and because its 2 asymmetric carbon atoms might render it more susceptible to configurational modification by racemization, a ready production of threonine deficiency was considered likely. This was confirmed; addition of threonine to an autoclave hydrolysate (heated with 10 per cent sulfuric acid for 8 hours at 165°) promoted about as rapid growth as had the reflux hydrolysate which it replaced. Longer heating at higher temperatures produced deficiencies not fully met by threonine alone.

The present communication records growth data which sub-

* Part of this paper was presented in abstract before the American Society of Biological Chemists at New Orleans in March, 1940 (Borchers and Berg, 1940).

Some initial observations were presented in a dissertation submitted by John R. Totter in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

[1] Private communication from Dr. W. C. Rose.

stantiate these observations and summarizes results obtained by analysis of representative hydrolysates for threonine as chemical methods became available.

EXPERIMENTAL

Feeding Studies—In an attempt to confirm and extend our observation that hydrolysis of zein in the autoclave may induce deficiencies not remedied by the usual supplements, four hydrolysates were compared for their effect on growth. These were prepared by refluxing with 25 per cent sulfuric acid solution for 18 hours (Hydrolysate R); by heating in the autoclave at 165° for 8 hours with 10 per cent sulfuric acid solution (Hydrolysate A-165); by autoclaving at 140° for 8 hours with 14 per cent sulfuric acid (Hydrolysate A-140); and by heating in the autoclave at 180° for 15 hours with 14 per cent sulfuric acid (Hydrolysate A-180). Hydrolysate A-140 was chosen because it showed nearly maximum free amino nitrogen and much less decomposition than Hydrolysate A-165; Hydrolysate A-180, because decomposition in it was extensive. The hydrolysates were rendered suitable for feeding by removing the sulfate ion exactly and concentrating to dryness, essentially as directed by Berg and Rose (1929). Unhydrolyzed zein served as a control. Each diet contained zein or zein hydrolysate 14.8 per cent, $l(+)$-lysine dihydrochloride 0.75, $l(-)$-tryptophane 0.2, $l(-)$-cystine 0.2, $l(+)$-histidine monohydrochloride 0.37, sodium bicarbonate 0.73, agar 2.0, salt mixture (Hubbell, Mendel, and Wakeman, 1937) 2.5, sucrose 15.0, starch 39.45, cod liver oil 5.0, and Crisco 19.0. The sodium bicarbonate was added to neutralize the hydrochlorides of lysine and histidine. The amino acids used here and for later supplementation were prepared in this laboratory; subsequent supplements replaced equal weights of the zein or of the hydrolysate. The vitamin B complex was fed separately in the form of two pills daily, each containing 0.02 mg. of crystalline riboflavin, 0.02 mg. of thiamine chloride, 0.1 mg. of nicotinic acid, 25 mg. of ryzamin-B, and enough starch to provide a consistency suitable for molding.

The experimental animals were litter mate rats weighing initially 44 to 64 gm.; they were housed in individual false bottomed cages; food and water were available at all times. To allow clear presentation of the growth data in a single chart

(Fig. 1), curves are given for only three of the five or six animals in each group; the records omitted were practically duplicates of those included. No striking difference was observed between average growth response on whole zein and on zein Hydrolysate R or A-140; individual growth rates varied widely, especially on unhydrolyzed zein. So far we have been only mildly successful in improving the nutritive value of whole zein by supplementing

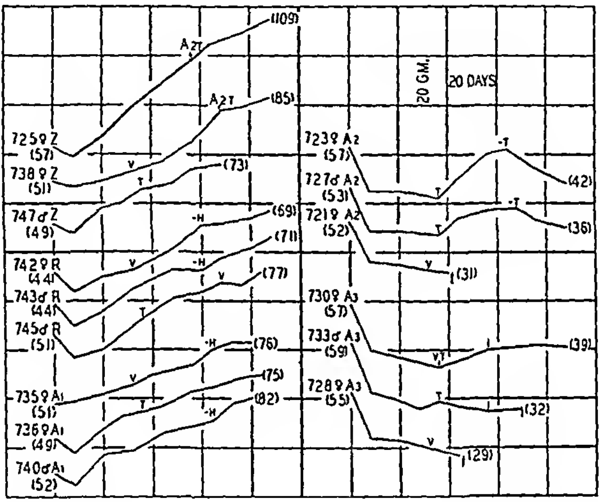

Fig. 1. Growth on zein and zein hydrolysates. Initial and final weights are given in parentheses. The daggers signify death. The symbols, Z, R, A_1, A_2, and A_3 represent basal diets containing zein, zein hydrolyzed under a reflux, and in the autoclave at 140°, 165°, and 180°, respectively. On the two upper left curves the A_2 which succeeds Z denotes replacement. Supplementation of the basal diet with *dl*-threonine (0.6 per cent) is indicated by T, with *dl*-valine (0.84 per cent) by V, and with *dl*-isoleucine (0.5 per cent) by I. Subsequent withdrawal is indicated by the minus sign. Removal of the histidine contained in the basal diet is represented by $-H$.

it with essential amino acids; normal rate of growth has not yet been obtained. Addition of threonine to any one of these diets produced no distinct growth acceleration.

On Hydrolysate A-165 all animals showed marked loss in weight; when threonine was added, growth began promptly at a rate approximately the same as that observed on pure zein or on Hydrolysate R or A-140. When the diet containing Hydrolysate A-165,

to which threonine had been added, was fed to rats which had previously received whole zein, growth continued without interruption at a rate only slightly reduced. Hence, under the experimental conditions obtaining, threonine effectively met the nutritional deficiency induced by the autoclaving. Tests with a casein hydrolysate prepared like Hydrolysate A-165 gave similar results.

Rats on Hydrolysate A-180 also showed marked losses in weight; when threonine was added, three of the four animals still living responded by growing, but at a rate decidedly below the average for the series receiving Hydrolysate A-165. Lack of adequate threonine was evidently not the only nutritional deficiency induced by this more drastic autoclaving treatment; additional supplements of isoleucine or valine caused no further improvement.

Differences in growth response on the hydrolysates thus correlated well with differences in optical rotation and in amino nitrogen content, as summarized in Table I. The extent of amino acid destruction was indicated also by ammonia content. In Hydrolysate A-165, 28 per cent of the nitrogen was found in this form before removal of the sulfate and evaporation to dryness. The ammonia content of Hydrolysates R, A-140, and A-180 was not determined, but hydrolysates prepared under the same conditions contained about 19, 22, and 30 per cent of ammonia nitrogen, respectively, before concentration.

Estimations of Threonine Content—When the method of Block and Bolling (1939) became available for estimating threonine, we undertook to apply it to determine the degree of threonine destruction in the hydrolysates fed. This method is empirical and unsuited to casual application. After considerable study several convenient modifications were found possible. These consisted chiefly of the elimination of the 4° bath, the preparation of the aldehyde-free glacial acetic acid-lead tetraacetate mixture in a single step, omission of the precipitation of the sulfate ion from the hydrolysates, and the use of aqueous standards of threonine. Results obtained upon incorporating these changes in the procedure were essentially the same as those obtained with the unmodified method. In our experience the rate of aeration seemed especially significant; since both too slow and too rapid speeds yielded less than maximum color and no set rate was suitable for all analyses,

it was necessary to establish an optimum rate for each test. After some experience with the method as a whole, maximum errors on separately run aliquots were reduced to 5 per cent or less.

Publication of the data obtained with the Block and Bolling procedure has been delayed to allow simultaneous presentation of results obtained with the recent method of Shinn and Nicolet (1941). Their procedure is relatively simple. It requires no standard and reagents obtainable on the market are employed. For our initial tests periodic acid was not immediately available; substitution of potassium periodate in N sulfuric acid and modification of the other solutions, to permit neutralization of the extra acidity and to keep the final total volume approximately the same, gave results essentially identical with those obtained later with the procedure as published. An average of 96.8 per cent of the theoretical yield of acetaldehyde has been produced from several samples of threonine; this agrees with the findings of the originators.

Data obtained by applying the two threonine methods to the same samples of threonine and allothreonine[2] and to the same hydrolysates of casein and zein are summarized in Table I.

The Shinn and Nicolet procedure yielded as much acetaldehyde from allothreonine as from threonine. Identical yields from the *dl* forms of these amino acids were obtained also by Martin and Synge (1941) whose procedure is similar in principle but produces less acetaldehyde. In the Block and Bolling procedure less than half as much color was produced from allothreonine as from the standard *dl*-threonine during 1 hour of aeration; in the 2nd hour an additional 10 per cent could be accounted for. Calculations based on the response of threonine and allothreonine to the two methods suggest that the mixture of threonine and allothreonine

[2] The nomenclature is that of Meyer and Rose (1936) who designated natural threonine $d(-)$ because it is structurally analogous to $d(-)$-threose. We are indebted to Dr. H. E. Carter for the samples of $l(+)$-threonine and *l*-, *d*-, and *dl*-allothreonine used in these studies. The synthetic mixture of *dl*-threonine and *dl*-allothreonine and the samples of $d(-)$- and *dl*-threonine were prepared in this laboratory by Dr. C. D. Bauer who used the synthetic and resolution procedures of West and Carter (1937). All of the samples were carefully dried to remove any trace of alcohol, since such might undergo conversion to acetaldehyde and thus be a source of error.

obtained by synthesis according to the method of West and Carter (1937) contained 96 per cent of these amino acids, of which 35 or 36 per cent was threonine. This is in general agreement with the observation made by West and Carter that the mixture of α-bromo-

TABLE I

Analysis of Threonine and Allothreonine and of Casein and Zein Hydrolysates

	α_D*	$[\alpha]_D^{20}$†	N found‡	Threonine found	
				Block-Bolling method	Shinn-Nicolet method
	degrees	*degrees*	*per cent*	*per cent*	*per cent*
d(−)-Threonine		−27.4	11.03	100.0	96.4
l(+)-Threonine		+28.1	11.53	98.7	97.7
dl-Threonine			11.71	101.5	96.6
d-Allothreonine		+7.9	11.64	47.5	
l-Allothreonine		−8.2	11.52	43.0	
dl-Allothreonine			11.52	43.0	96.9
Threonine-allothreonine mixture, as synthesized			10.98	65.0	93.1
Casein Hydrolysate R				3.5	3.2
" " A-165				1.0	0.7
Zein Hydrolysate R	+2.6		72	2.4	2.20
" " A-140	+2.0		69	1.9	1.65
" " A-165	+0.5		60	0.5	0.65
" " A-180	+0.3		58	0.15	0.25

* The rotations of the zein hydrolysates were read in 1 dm. tubes before neutralization of the sulfuric acid and are based on concentrations of 27.5 mg. of total nitrogen per cc.

† In the determination of the specific rotations of threonine and allothreonine, 2 per cent aqueous solutions (0.2 gm. in 10 cc.) were polarized in 2 dm. tubes.

‡ The nitrogen figures for threonine and allothreonine represent total nitrogen by the micro-Kjeldahl method; calculated nitrogen is 11.76 per cent. The nitrogen figures for the zein hydrolysates refer to amino nitrogen expressed as per cent of total nitrogen. The latter determinations were made prior to removal of the sulfuric acid used for hydrolysis.

β-methoxy-*n*-butyric acids prepared in their method of synthesis contains from 30 to 40 per cent of the precursor of *dl*-threonine.

The data for the threonine content of the zein and casein hydrolysates in Table I are not corrected for alanine, of which zein contains almost 10 per cent and casein about 2. The Block and Bolling procedure, applied to 2 mg. samples of alanine (ap-

proximately the amount present in the zein samples analyzed), yielded a little less than 2 per cent of the color obtainable from a molecularly equivalent amount of threonine. Correction on this basis would lower the estimated threonine content of zein Hydrolysate R from 2.4 to about 2.2 per cent, but would have little effect on the estimate on casein Hydrolysate R. Just what correction should be applied to the analyses of the autoclaved hydrolysates is uncertain because the extent to which alanine may have been destroyed is not known. In the Shinn and Nicolet procedure only 0.18 per cent as much iodine was required to titrate the blank from 40 mg. samples of commercial *dl*-alanine as would have been needed had a molecularly equivalent weight of threonine been analyzed. Hence correction for alanine in this method seems pointless. Calculations to compensate for the production of but 97 per cent of the theoretical amount of acetaldehyde might well be applied, but this has not been done. Correction of the data which Martin and Synge (1941) obtained by applying their method to zein, and which they estimate to be 30 to 35 per cent low, gives a threonine content of 2.16 to 2.24 per cent.

Despite discrepancies in the figures for the threonine content, with both the Block and Bolling and the Shinn and Nicolet methods decreases of the same order are shown in the autoclave hydrolysates: about 20 per cent in Hydrolysate A-140, 70 per cent in Hydrolysate A-165, and 90 per cent in Hydrolysate A-180. Lack of information concerning the degree of alanine destruction prevents drawing definite conclusions concerning allothreonine production. That there may be such in Hydrolysates A-165 and A-180 is suggested by the lower estimate with the Block and Bolling than with the Shinn and Nicolet procedure.

Pure threonine also undergoes marked destruction when autoclaved in sulfuric acid solution; preliminary tests have been inadequate in scope to clarify interrelationships between destruction and racemization and allothreonine formation. Preparation of an adequate supply of the optical isomers of threonine for use in such studies is in progress.

SUMMARY

Feeding tests show that sulfuric acid hydrolysates of zein prepared under a reflux or under relatively mild conditions in the autoclave possess about the same capacity as zein to support

growth when incorporated in diets supplemented with lysine, tryptophane, histidine, and cystine. More drastic autoclave treatment produces hydrolysates which do not support growth under similar conditions unless threonine is added; still more severe autoclaving induces deficiences which cannot be met by threonine supplementation alone.

Threonine and allothreonine produce equal amounts of acetaldehyde in the procedure of Shinn and Nicolet; in the method of Block and Bolling considerably less color is produced with allothreonine than with threonine. Assays of the several hydrolysates of zein and casein according to either method show that threonine is destroyed as the conditions of hydrolysis become more severe. Pure threonine also suffers marked destruction when autoclaved with sulfuric acid under conditions which destroy it in zein.

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THE KINETICS OF THE HYDROLYSIS OF UREA AND OF ARGININE

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This investigation of the hydrolysis of urea and of arginine was undertaken in connection with a study of ammonia formation from proteins in alkaline solution.

Hydrolysis of Urea and Cyanate

Fawsitt (1) and Werner (2) have studied the hydrolysis of urea by acids and by alkalies. They agreed in assuming that cyanate was an intermediate in the hydrolysis, but differed widely as to the details of the mechanism and as to experimental values for the velocity constants of the reactions involved.[1] The reverse reaction, the synthesis of urea from ammonia and cyanate, has been investigated in some detail by Walker and his coworkers (3) and more recently by Warner and Stitt (4) and by Svirbely and Warner (5). The latter workers have concluded, on the basis of the effect of ionic strength on the velocity, that the reaction is one between ammonium and cyanate ions.

Because of the unsatisfactory nature of the data on the kinetics of the hydrolysis of urea, we have reinvestigated the rates and mechanism of the reactions involved. We were chiefly interested in the hydrolysis by alkali, but the work was extended to include neutral and acid pH ranges.

[1] Werner states that the lack of agreement between his results and those of Fawsitt on the rate of hydrolysis of urea was due to the fact that his experiments were performed under a reflux, whereas Fawsitt's reaction mixtures were heated in sealed tubes. Contrary to Werner's contention, we have found no difference in the rate of hydrolysis in an alkaline reaction mixture, whether heated in sealed tubes or under a reflux.

Methods

Reaction mixtures were prepared by adding measured quantities of HCl, NaOH, or chosen buffer solutions to standard solutions of urea or of potassium cyanate. A series of samples of each reaction mixture was sealed in small Pyrex tubes which were heated in a boiling water bath (100°) or a boiling methyl alcohol bath (66°). Tubes were removed at various time intervals, chilled in ice water, and the contents analyzed by the methods described below.

Ammonia was determined by the method of Conway and Byrne (6). The presence of cyanate in the reaction mixture does not interfere with this analysis. Ammonia plus cyanate was determined by acidifying an aliquot of the reaction mixture and allowing it to stand for 20 minutes, to convert the cyanate to ammonia and CO_2. The ammonia was then determined in the Conway unit. Urea was determined by the method of Allen and Luck (7). It was found necessary to avoid large excesses of HCl, NaOH, or neutral salt to obtain complete precipitation of the xanthydrol urea.

Determinations of all the reactants were not made in all experiments.

Results

All of the nitrogen in the reaction mixtures was accounted for as urea, ammonia, and cyanate. Possible side reactions which could lead to the formation of cyanuric acid or cyamelide can thus be disregarded. Structural relationships suggest carbamate as an intermediate, and the work of Lewis and Burrows (8) indicates that it has some significance in the equilibria which would ultimately obtain in a neutral solution of urea at 100°. The rate of attainment of the carbamate-carbonate equilibrium is, however, so great at 100° at any pH that carbamate would not be expected to accumulate in the reaction mixtures (9, 10). The detailed analysis presented below indicates that carbamate cannot be formed in large enough amounts to be of quantitative significance in determination of the course of the hydrolysis in dilute solution.

Thus the following two reactions can tentatively be assumed.

$$CO(NH_2)_2 \rightleftharpoons NH_4^+ + OCN^- \quad (1)$$

$$OCN^- + H_3O^+ \rightarrow NH_3 + CO_2 \quad (2)$$

This is a similar sequence of reactions to that postulated by Werner and Fawsitt in their studies on urea hydrolysis.

The reversible nature of Equation 1 in neutral solution has been well established (3–5). There is no evidence for the reversibility of Equation 2 under the conditions of these experiments.

On the basis of Equations 1 and 2, the following differential equations can be set up to describe the course of the hydrolysis

$$\frac{du}{dt} = -k_1 u + k_2 xy \tag{3}$$

$$\frac{dy}{dt} = k_1 u - k_2 xy + k_3 x \tag{4}$$

$$\frac{dx}{dt} = k_1 u - k_2 xy - k_3 x \tag{5}$$

where

u = concentration of urea
y = " " ammonia
x = " " cyanate
$2u + y + x$ = total concentration of nitrogen = a
k_1 = velocity constant of the forward component of Equation 1
k_2 = " " " " reverse reaction
k_3 = " " " Equation 2

These equations will hold at any pH, but the values of the constants may be expected to vary individually with the pH. In certain pH regions the equations can be modified to permit the ready derivation of the constants from the experimental data.

In acid solution (less than pH 5) the rapid hydrolysis of cyanate reduces x to so small a value that it is possible to neglect the k_2xy term completely. Levy[2] has made measurements on the hydrolysis of cyanate under these conditions at 25°. He found that $k_3 = 3.2$ at pH 4 and that it increases in direct proportion to (H^+), a double inflection in the curve being evident near the ionization constant of cyanic acid. The reaction is too fast to measure at higher temperatures and cyanate can thus be considered to be absent in our acid reaction mixtures. This sets an effective lower pH limit to the reversibility of Equation 1.

In alkaline solution (greater than pH 12) Equation 1 can also

[2] Levy, M., personal communication.

be considered to be irreversible. This was shown by experiments on the hydrolysis of cyanate in NaOH in which no urea was formed, the sole products being ammonia and carbonate. The effect of ionic strength on the velocity of the reverse of Equation 1 indicates that the reaction is one between NH_4^+ and OCN^- (4). These two considerations make it probable that an upper limit to the reversibility of the reaction is set by the dissociation $NH_4^+ = NH_3 + H^+$.

Below pH 5 or above pH 12, therefore, Equation 3 reduces to the first order equation

$$\frac{du}{dt} = -k_1 u \tag{6}$$

k_1 can be calculated either from the rate of disappearance of urea or the rate of formation of ammonia plus cyanate. The results at 100° and 66° will be found in Tables I and II respectively. The constants are calculated in all cases in natural logarithms and reciprocal hours.

Data were also obtained on the hydrolysis of KOCN in alkaline solution. For this situation Equation 5 reduces to a first order equation with respect to x, and k_3 can be calculated. The data obtained appear in Table III.

In alkaline solution where k_1 and k_3 are of similar magnitude, the concentration of cyanate at time intervals during the hydrolysis of urea can be calculated from the separately determined values of these constants. An effective test of the validity of Equations 1 and 2 under these conditions is the comparison of the calculated concentration of cyanate with the experimentally determined value. In alkaline solution, Equation 5 becomes $dx/dt = k_1u - k_3x$.

If this is combined with Equation 6 and integrated, the relation

$$x = \frac{ak_1}{2(k_3 - k_1)} \left(e^{-k_1t} - e^{-k_3t}\right) \tag{7}$$

is obtained.

In Table IV, x has been calculated from Equation 7 for each of the time intervals in a urea hydrolysis and is entered for comparison with the experimental value. The agreement is well within the experimental error. This calculation has been made

TABLE I

Urea Hydrolysis at 100°

k_1 was calculated from urea determinations by the xanthydrol method or from NH_3 + cyanate determinations by the Conway method, as indicated. The ionic strength was equal to the NaOH concentration, except in the cases noted.

Concentration NaOH	Method	μ	pH	k_1	Log k_1 + 2	Initial urea concentration
M						*M*
4.04	Xanthydrol		14.50	0.745	1.872	0.0625
3.24	Conway		14.37	0.534	1.728	0.050
2.83	Xanthydrol		14.29	0.438	1.641	0.040
2.43	Conway		14.21	0.345	1.538	0.025
2.01	Xanthydrol	2.01	14.10	0.313	1.495	0.020
2.02	"	3.02	14.15	0.322	1.508	0.040
2.02	"	4.02	14.20	0.352	1.547	0.040
1.52	Conway		13.98	0.239	1.379	0.025
1.48	"		13.95	0.230	1.362	0.050
1.00	Xanthydrol		13.76	0.206	1.314	0.010
0.750	Conway		13.64	0.185	1.267	0.025
0.732	"		13.63	0.182	1.269	0.050
0.501	Xanthydrol		13.47	0.178	1.251	0.005
0.405	Conway		13.39	0.167	1.223	0.025
0.371	"		13.35	0.168	1.224	0.025
0.252	"		13.20	0.166	1.220	0.025
0.244	Xanthydrol		13.18	0.165	1.218	0.005
0.241	"		13.18	0.170	1.231	0.005
0.240	Conway	1.24	13.18	0.157	1.196	0.025
0.192	"		13.08	0.157	1.197	0.025
0.105	"		12.83	0.155	1.190	0.025
0.0974	Xanthydrol	0.25	12.78	0.154	1.186	0.005
0.0968	"	0.25	12.78	0.150	1.176	0.01
0.0513	"	0.25	12.51	0.150	1.175	0.005
0.0510	"	0.25	12.50	0.153	1.186	0.005
0.0208	Conway		12.19	0.143	1.159	0.025
Acetate	Xanthydrol	0.25	4.01	0.144	1.159	0.005
Citrate	"	0.25	2.39	0.146	1.165	0.01
HCl						
0.0634	Xanthydrol	0.25	1.40	0.142	1.153	0.005
0.105	"	0.25	1.09	0.126	1.102	0.005
0.258	"		0.71	0.120	1.078	0.005
0.510	"		0.41	0.0885	0.947	0.01
1.016	"		0.08	0.0603	0.780	0.02
1.76	"		−0.22	0.0386	0.587	0.04
2.97	"		−0.59	0.0221	0.344	0.0625

Table II

Urea Hydrolysis at 66°

All values are recorded as in Table I.

Concentration NaOH	Method	μ	pH	k_1	Log k_1 + 4	Initial urea concentration
M						*M*
4.04	Xanthydrol	4.04	14.50	0.0386	2.587	0.0625
3.14	Conway	3.14	14.35	0.0228	2.358	0.025
1.57	"	1.57	13.98	0.00802	1.904	0.025
0.792	"	0.79	13.66	0.00363	1.560	0.025
0.315	"	0.31	13.28	0.00237	1.364	0.025
0.105	"	0.10	12.84	0.00209	1.321	0.025
0.0513	Xanthydrol	0.25	12.50	0.00205	1.311	0.005
0.0102	"	0.25	11.80	0.00189	1.277	0.005
HCl						
0.0105	Xanthydrol	0.25	2.10	0.00213	1.328	0.005
0.0530	"	0.25	1.39	0.00217	1.336	0.005
0.0981	"	0.25	1.13	0.00236	1.373	0.005
0.490	"	0.49	0.43	0.00154	1.187	0.01
1.47	"	1.47	−0.12	0.000934	0.970	0.04
1.69	"	1.69	−0.20	0.000743	0.871	0.04
3.14	"	3.14	−0.74	0.000316	0.500	0.0625

Table III

Hydrolysis of Potassium Cyanate

The three values in phosphate buffers were indirectly obtained, as explained in the text.

Temperature	NaOH concentration	k_3
°C.	*M*	
100	1.48	0.170
	0.40	0.175
	0.40	0.177
	0.37	0.178
	0.100	0.179
	0.100	0.182
	0.0968	0.172
66	0.25	0.0055
	Phosphate buffer	
	pH	
100	7.55	1.7
	6.95	3.2
	6.30	13.0

for a number of other experiments at different hydroxyl ion concentrations with equally good results. It can thus be concluded that carbamate or other possible intermediates are not quantitatively significant in determining the course of urea hydrolysis under these conditions.

The treatment of the data obtained between pH 5 and 12 is more difficult. As was previously stated, no urea is formed during the hydrolysis of cyanate in NaOH of a concentration of 0.1 N or greater. As the pH is progressively decreased, urea begins to accumulate in the reaction mixtures. This formation of urea is apparent in 0.01 N NaOH and increases to a maximum at about pH 8. Below this pH the formation of urea again decreases as the rate of hydrolysis of cyanate becomes greater. Since the ionization constant of NH_4^+ is about 8.7×10^{-10} at 100° (11), this is consistent with the interpretation that only NH_4^+ participates in the reverse of Equation 1.

TABLE IV

Urea Hydrolysis at 100° in 0.0968 N NaOH

Urea, NH_3, and NH_3 + cyanate were determined. The observed cyanate was obtained by difference; calculated cyanate was obtained from Equation 7, with $k_1 = 0.150$ and $k_2 = 0.172$. The total nitrogen concentration was calculated from the urea and NH_3 + cyanate determinations.

Time	Urea	NH_3	NH_3 + cyanate	Observed cyanate	Calculated cyanate	Total nitrogen concentration
hrs.	*M*	*M*	*M*	*M*	*M*	*M*
0.00	0.0100	0.00	0.00	0.00	0.00	0.0200
1.00	0.00852	0.00153	0.00283	0.00130	0.00123	0.0199
2.00	0.00726	0.00324	0.00532	0.00218	0.00225	0.0199
3.00	0.00631	0.00457	0.00723	0.00266	0.00280	0.0198
4.30	0.00524	0.00605	0.00924	0.00319	0.00321	0.0197
5.75	0.00419	0.00802	0.01131	0.00329	0.00341	0.0197

At pH values at which the ammonia is partially or completely present as the free base, a fraction of it will be in the small gas phase in the sealed tubes. For this reason and because of uncertainties in the ionization constant of NH_4^+ at 100° no attempt has been made to account quantitatively for the data in the pH region 8 to 12.

Below pH 7.5 the ammonia is completely ionized and the course of the reaction should be described by Equations 3, 4, and 5. Unfortunately, these equations cannot be integrated as they stand, and a numerical method of solving them must be resorted to. The method adopted was one outlined by Levy and Baggott (12). It is not suited to the estimation of the constants, k_1, k_2, and k_3. Consequently these must be obtained independently.

The results of calculations on an experiment at pH 6.95 are shown in Fig. 1. The data, which include hydrolyses of both

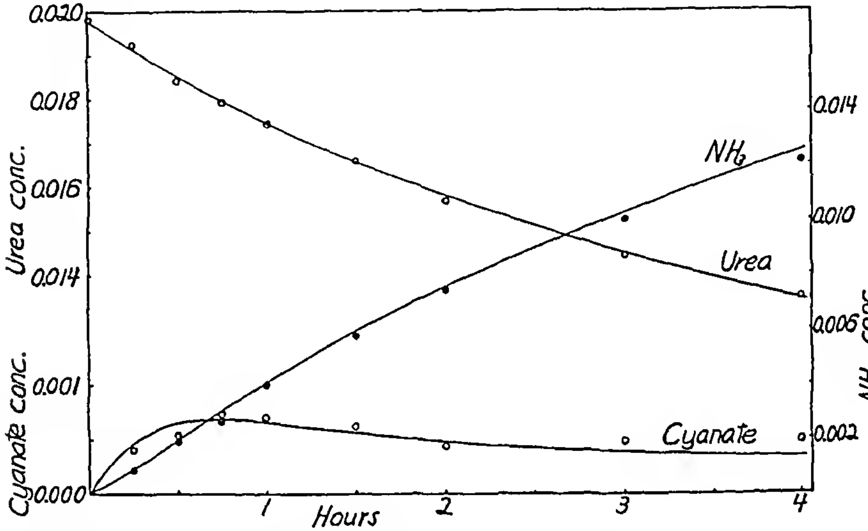


FIG. 1. Hydrolysis of urea in a phosphate buffer, pH 6.95, ionic strength 0.25, at 100°. Concentrations are expressed as moles per liter. See the text for explanation of the curves.

urea and cyanate, are recorded in Table V. The values of the constants were estimated as follows:

From the plot of log k_1 against pH in Fig. 2, it can be seen that a plausible extrapolation through the neutral region is a straight line parallel to the pH axis. This makes $k_1 = 0.147$ between pH 2 and 12. Approximate confirmation of this can be obtained by calculating first order constants from the urea data in this experiment for time intervals of less than 1 hour.

A value for k_2 can be obtained from the work of Warner (4, 5). His measurements include determinations of k_2 as a function of ionic strength in the temperature range 30–80°. k_2 was cal-

culated to be 240 at 100° and $\mu = 0.25$ from the value at 70° and the critical increment over the temperature range studied.

An approximation to k_3 can be obtained from the experiment on the hydrolysis of potassium cyanate. From the data in Table V it can be seen that the concentration of urea formed is

TABLE V

Hydrolysis of Urea and Cyanate in Phosphate Buffer at pH 6.95 at 100°

All values are recorded as in the preceding tables.

Ionic strength = 0.25.

Urea hydrolysis

Time	Urea	NH_3	NH_3 + cyanate	Cyanate	Total nitrogen concentration
hrs.	*M*	*M*	*M*	*M*	*M*
0.00	0.01982	0.00	0.00	0.00	0.0396
0.25	0.01925	0.00088	0.00128	0.00040	0.0398
0.50	0.01840	0.00195	0.00249	0.00054	0.0394
0.75	0.01788	0.00264	0.00338	0.00074	0.0391
1.00	0.01745	0.00400	0.00470	0.00070	0.0396
1.50	0.01662	0.00580	0.00642	0.00062	0.0397
2.00	0.01570	0.00750	0.00794	0.00044	0.0393
3.00	0.01444	0.01003	0.01051	0.00048	0.0394
4.00	0.01345	0.01213	0.01261	0.00048	0.0395

Cyanate hydrolysis

Time	NH_3	Urea	$\frac{k_3}{k_2}$	k_3
hrs.	*M*	*M*		
0.167	0.00229	0.00030	0.01	2.4
0.333	0.00364	0.00062	0.013	3.1
0.667	0.00499	0.00123	0.014	3.4
1.00	0.00540	0.00148	0.014	3.4
1.50	0.00588	0.00151	0.015	3.8

sufficiently small for time intervals up to 1 hour so that its subsequent hydrolysis (k_1u term) can be neglected as a first approximation.

Equation 4 is divided by Equation 5, the k_1u term being neglected, and integrated. After substitution of $2u + y = a - x$, the relation

$$u + y = \frac{k_3}{k_2} \ln \frac{k_3/k_2}{k_3/k_2 - y} \tag{8}$$

is obtained.

With the experimental values for u and y, k_3/k_2 was calculated for each of the time intervals, solution being made by approximation. The values obtained are entered in Table V together with those of k_3 calculated by assuming $k_2 = 240$.

With these values for the constants, the equations were solved numerically to give u, x, and y at various time intervals. The

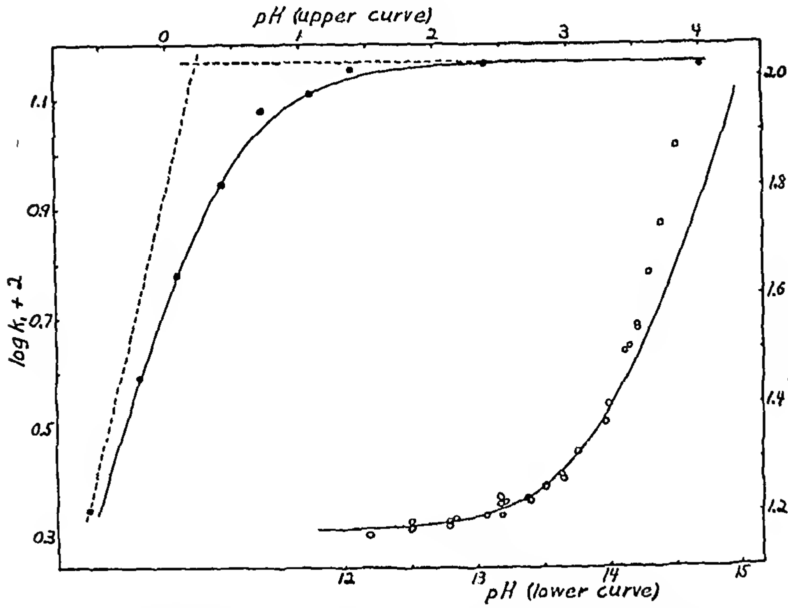


FIG. 2. Hydrolysis of urea as a function of pH at 100°. The dash line curves are the asymptotes approached by the solid line curves. Their intersection indicates the value of pK.

effect of varying k_3 within the limits shown in Table V was tried and $k_3 = 3.2$ was found to give the best fit with the data. The values obtained were plotted to give the smooth curves in Fig. 1. The experimental determinations for the urea hydrolysis are shown as the discrete points in the graph (Fig. 1).

The deviations of the experimental points for NH_3 and urea from the calculated curves may be due to the small changes in the pH of the reaction mixtures as the hydrolysis proceeded, or they might be accounted for by further small adjustments in the

values for k_3. The cyanate determinations are less accurate than the others because of the "difference" method employed in the determination. Equally good agreement was obtained in an experiment at pH 7.55 with the same values for k_1 and k_2 and with $k_3 = 1.7$.

The mechanism assumed in Equations 1 and 2 will thus adequately account for the reaction under all conditions of pH.

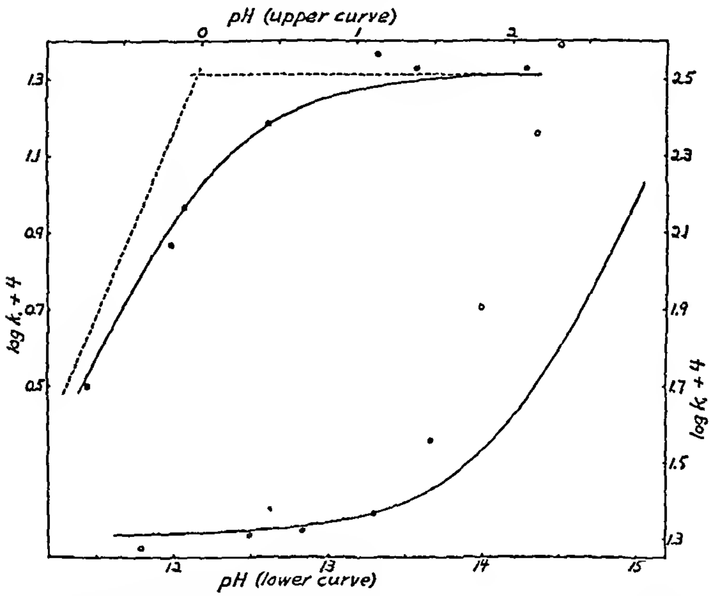


FIG. 3. Hydrolysis of urea as a function of pH at 66°. See legend to Fig. 2 for explanation of the dash line.

Reaction Mechanism

Figs. 2 and 3 show a plot of log k_1 against pH at 100° and 66° respectively. pH was calculated from the concentration of HCl or NaOH and the activity coefficient data of Randall and Young (13) for HCl, and of Harned (14) for NaOH. It was assumed that $pK_w = 13.93$. These calculations give the pH at 25°. No attempt has been made to calculate the pH at the experimental temperatures. Any temperature correction for pH will change the shape of the curves only in so far as the activity coefficients change with the temperature. However, there would be a con-

siderable displacement of the alkaline end of the curve on the pH axis if such correction were made.

The curve shows that between pH 2 and 12 k_1 is independent of (H^+). Below pH 2 and above pH 12, there are inflections in the curve, each of which indicates inverse proportionality of k_1 to (H^+). The inflection in acid solution is in the region of the ionization constant of urea. This constant (K_w/K_b) was determined by Walker and Wood (15) to be about 0.7 at 25°. The inflection in alkaline solution does not correspond to any known ionization of urea.

The log k_1-pH curve is consistent with an uncatalyzed reaction (or a water catalysis) between pH 2 and 12. Superimposed on this is a hydroxyl ion catalysis which becomes rate-controlling above pH 13. The drop in log k_1 below pH 2 can be accounted for by assuming a rate of hydrolysis of the urea ion which is negligible in comparison with that of the neutral molecule. These assumptions can be expressed in the following equations.

$$(NH_2)_2CO = NH_4^+ + OCN^- \tag{9}$$
$$(NH_2)_2CO + OH^- = NH_4^+ + OCN^- + OH^- \tag{10}$$
$$(NH_2)_2COH^+ = NH_4^+ + OCN^- + H^+ \tag{11}$$

From these equations, the following expression can be formulated for the observed rate of reactions, k_1, in terms of the intrinsic rates, a, b, and c, of Equations 9, 10, and 11, respectively.

$$k_1[(U) + (U^+)] = a(U) + b(U)oh + c(U^+) \tag{12}$$

where (U) = concentration of the neutral urea molecule
and (U^+) = " " " positive urea ion
oh and h = the activities of hydroxyl and hydrogen ions respectively

(U) and (U^+) are related by the equation

$$K = \frac{(U)h}{(U^+)} \tag{13}$$

If we combine Equations 12 and 13 and neglect the $c(U^+)$ term

$$k_1 = a\frac{K}{h + K} + b\frac{K}{h + K}\frac{K_w}{h} \tag{14}$$

In the region where b is rate-controlling, $K/(h + K) = 1$ and Equation 14 can be written

$$k_1 = a\frac{K}{h+K} + b\frac{K_w}{h} \tag{15}$$

The constants a and b can be derived from the experimental data. Between pH 2 and 12, a is the rate-controlling constant. In this region $K/(h + K) = 1$ and $k_1 = a$. At 100° the average value for k_1 is 0.147. With this value for a, $b = 0.0826$ was found to give the best fit with the data in alkaline solution. Similarly $K = 0.58$ was derived from the data in acid solution. The solid lines in Fig. 2 are plotted from the equation

$$k_1 = 0.147\frac{K}{h+K} + 0.0826\frac{K_w}{h} \tag{16}$$

where $K = 0.58$.

The experimental points show marked deviations from this equation only in strongly alkaline solution in which the observed rate rises more rapidly than would be predicted from the equation. No other mechanism could be found which would give a more satisfactory account of this part of the curve.

The data at 66° were treated similarly and the equation

$$k_1 = 0.00205\frac{K}{h+K} + 0.00115\frac{K_w}{h} \tag{17}$$

where $K = 1.07$ was derived. The solid lines in Fig. 3 are plotted from this equation. The deviations in alkaline solution are more marked in this case than at 100°.

There are several alternate formulations of the reaction mechanism which would result in a dependence of k_1 on pH, formally equivalent to that described by Equation 14. Assumptions such as a reaction between U^+ and OH^- in acid solution, or the involvement of a hypothetical urea anion in alkaline solution, cannot be excluded. The mechanism suggested above seems to be the most plausible.

Influence of pH on k_3

Table III contains the observed values for k_3 in alkaline solution, together with the values at pH 6.95 and 7.55, which were derived as previously described, and a value at pH 6.30 obtained by the same method.

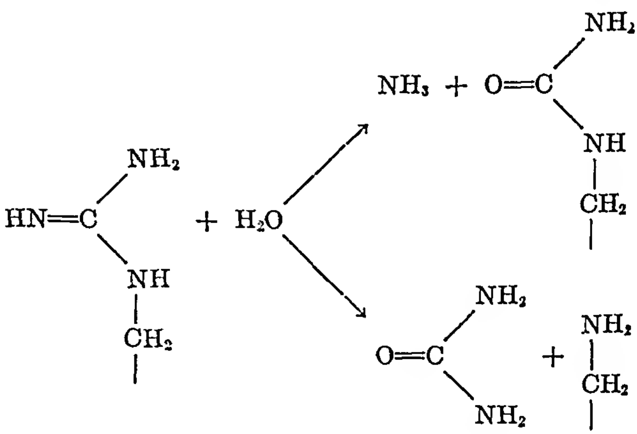
The three constants obtained in buffer solutions show an approximate proportionality to (H^+). In NaOH solution, k_3 is independent of pH. These observations, together with those of Levy,[2] at 25° indicate that a hydrogen ion catalysis of the reaction is rate-controlling below about pH 8 and that a water catalysis becomes rate-controlling above this pH. These assumptions lead to an equation of the type $k_3 = k'h + k''$, where k' and k'' are the intrinsic constants for the hydrogen ion and the water catalysis respectively.

There are insufficient data to derive accurate values for the constants in the equation.

Hydrolysis of Arginine

Arginine is known to be stable in acid solution and survives long periods of boiling with strong acid during the hydrolysis of proteins. Concentrated alkali hydrolyzes arginine with the production of 2 equivalents of ammonia. This fact is the basis of the Van Slyke procedure for the estimation of arginine (16).

The course of the reaction has been indicated by the work of Hellerman and Stock (17) who followed the hydrolysis in 0.1 N NaOH at 37°. They measured ammonia and urea and found a constant proportion between the two. Arginine disappeared according to a first order equation. They interpreted these results to mean that either of the C—N bonds in the guanidine part of the molecule could be hydrolyzed. The hydrolysis to form urea occurred at the greater rate. The other products of the reaction must have been ornithine and citrulline. We have



(18)

(19)

extended this analysis to include the effects of pH and temperature. The methods used were the same as in the urea experiments.

From Hellerman and Stock, it is assumed that arginine can be hydrolyzed in two ways, each reaction following a first order course with respect to arginine. These reactions can be represented by Equations 18 and 19.

Urea, of course, will be further hydrolyzed, but this reaction is sufficiently slow that it can be neglected at temperatures of 66° and below, up to about 50 per cent completion of the arginine hydrolysis. The citrulline will also be hydrolyzed but at a lower rate than urea, as is shown below. It can likewise be neglected.

Equations 18 and 19 can be described as follows:

$$\frac{du}{dt} = k_u z \tag{20}$$

$$\frac{dy}{dt} = k_y z \tag{21}$$

$$\frac{dz}{dt} = -kz \tag{22}$$

where

z = concentration of arginine
u = " " urea
y = " " ammonia
a = original arginine concentration $= z + u + y$

Equation 22 integrates to give

$$kt = \ln \frac{a}{z} = \ln \frac{a}{a - (u + y)} \tag{23}$$

from which k can be calculated. If Equation 20 is divided by Equation 21 and integrated, $u/y = k_u/k_y = m$.

If Equations 20 and 21 are added and $(a - z)$ substituted for $(u + y)$, it is evident that $k = k_u + k_y$. The system can thus be described by the two constants k and m and the others can be derived, if desired.

The results obtained at 66° and 35° are assembled in Table VI.

The reaction mixtures were made up from standard NaOH and arginine monohydrochloride. The NaOH concentration given in Table VI was calculated by allowing 1 equivalent for

TABLE VI

Hydrolysis of Arginine by NaOH at 66° and 35°

Temperature	NaOH	μ	Calculated pH	k	Log k + 2	Average m	Variation in m during experiment	Initial arginine concentration
°C.	M							M
66	0.100	0.150	12.66	0.0584	0.766	5.65	5.1–6.3	0.05
	0.127	0.177	12.77	0.0665	0.823	5.75	5.0–6.9	0.05
	0.393	0.443	13.32	0.145	1.161	5.57	5.1–6.6	0.05
	1.058	1.108	13.77	0.194	1.288	5.25	4.7–6.8	0.05
	2.35	2.40	14.18	0.205	1.312	5.16	4.8–5.9	0.05
	4.03	4.09	14.49	0.209	1.321	5.5	5.0–6.1	0.05
					Log k + 3			
35	0.185	0.224	12.99	0.00455	0.667	6.43	6.3–6.6	0.039
	0.936	0.975	13.71	0.00886	0.948	6.18	5.3–6.8	0.039
	4.00	4.08	14.49	0.00944	0.975	5.46	4.1–6.6	0.078

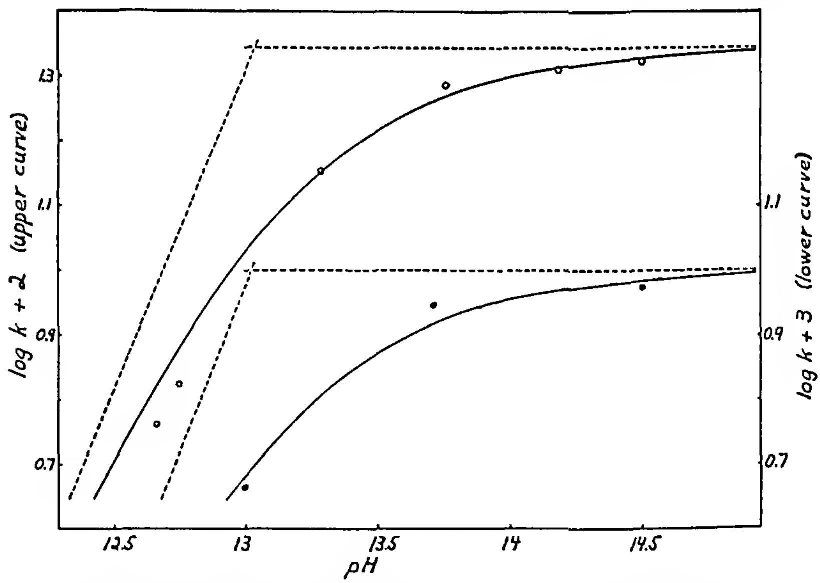


FIG. 4. Hydrolysis of arginine as a function of pH at 100° (upper curve, left-hand ordinates) and at 66° (lower curve, right-hand ordinates). See legend to Fig. 2 for explanation of the dash line.

neutralization of the hydrochloride. The pH of reaction mixtures was calculated approximately by assuming pK'_3 for arginine =

12.5 and by using the data of Harned (14) for the activity coefficient of NaOH. No attempt was made to correct the pH for the change in temperature.

There was some variation in m as the hydrolysis proceeded, and the limits in this variation in each experiment are shown in Table VI. There are no consistent changes in m with pH. Consequently, consideration of the effect of pH on the reaction can be limited to k.

A plot of log k against pH is shown in Fig. 4. The inflection in the curve occurs at about pH 13, which is somewhat higher than pK'_3 for arginine. However, it seems likely that this ionization is responsible for the inflection. No definite decision can be made between alternative possible mechanisms. In view of the stability of arginine at lower pH, the most direct description

TABLE VII

Hydrolysis of Citrulline by NaOH at 100° and 66°

Temperature	NaOH concentration	k_4
°C.	M	
100	0.124	0.0143
	1.12	0.0180
	3.18	0.0646
66	1.40	0.0011
	3.18	0.0028

of the log k-pH curve results from the following assumptions: (*a*) the rate of hydrolysis of the un-ionized guanidine group is independent of the pH; (*b*) the rate of hydrolysis of the charged guanidine group is negligible in comparison to (*a*).

The assumptions can be expressed in Equation 24

$$k = k' \frac{K}{h + K} \tag{24}$$

where k' is the intrinsic rate constant for the hydrolysis of the guanidine group and K is the ionization constant for this group.

The best fit with the data is obtained at 66° with $k' = 0.221$ and $K = 9.34 \times 10^{-14}$, and at 35° with $k' = 0.010$ and the same value for K. The solid curves in Fig. 4 are plots of Equation 24 with the above constants.

Hydrolysis of Citrulline

A few experiments on the hydrolysis of citrulline at 100° and 66° were performed in order to give complete account of the ammonia formed during the hydrolysis of arginine. Citrulline was prepared from arginine by the method of Fox (18). The experiments were carried out as previously.

First order constants for the formation of ammonia in three experiments at 100° and two at 66° were derived from Equation 25,

$$k_4 t = \ln \frac{a}{(\text{citrulline})} = \ln \frac{a}{a - (NH_3)} \tag{25}$$

At the longer time intervals in all of the experiments, there was some tendency for a decrease in rate below that required for a first order reaction. No urea could be detected in any of the samples. No analyses for cyanate were made, although it seems possible from the structure of citrulline that some would be formed. The data are recorded in Table VII.

The rate of hydrolysis shows some increase at the higher NaOH concentration. The increase is not in direct proportion to the hydroxyl ion concentration. This indicates that there may be an inflection in the log k-pH curve that would be evident on a more extended investigation of the reaction.

The low rate of hydrolysis of citrulline provides the reason for the drastic treatment of arginine required by the Van Slyke method of analysis (16).

The author is indebted to Professor R. K. Cannan for his advice and criticism during the course of this work and to Dr. M. Levy for permission to quote from unpublished data.

SUMMARY

1. Data are presented on the rate of hydrolysis of urea, cyanate, arginine, and citrulline under various conditions of pH and temperature.

2. Urea is hydrolyzed to ammonia and cyanate. This reaction is reversible at pH values at which ammonia is ionized. The reversibility is limited in acid solution by the rapid hydrolysis of cyanate.

3. Cyanate is hydrolyzed to ammonia and carbon dioxide. The

rate of hydrolysis is independent of pH in alkaline solution, but increases rapidly below about pH 9.

4. The assumption of cyanate formation as the only intermediate step in urea hydrolysis quantitatively accounts for the course of the reacion at all pH values.

5. Arginine is hydrolyzed by two simultaneous reactions: one producing ammonia and citrulline; the other, urea and ornithine. Each reaction is of the first order with respect to arginine concentration.

6. The change in the rate of hydrolysis of the above substances with respect to pH is discussed.

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THE FORMATION OF AMMONIA FROM PROTEINS IN ALKALINE SOLUTION

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Considerable amounts of ammonia may be formed during the alkaline hydrolysis of proteins. Osborne, Leavenworth, and Brautlecht (1) were of the opinion that the amide and guanidine groups of the protein were the sole source of this ammonia.[1] In the alkaline hydrolysis of wheat gliadin, however, Vickery (2) observed a slow formation of ammonia from unidentified sources. The significance of the observation was masked by the unusually large amount of amide nitrogen in this protein. In the report which follows, we present evidence that considerable amounts of ammonia may originate in groups other than amide and guanidine. A part of this may plausibly be attributed to partial deamination of cystine (3, 4), histidine (5), and serine (6). The evidence indicates, however, that a notable part—and that liberated under quite mild conditions of hydrolysis—originates in some structural feature of the protein itself, rather than in any specific amino acid component. The proteins studied were crystalline preparations of egg albumin, edestin, and β-lactoglobulin.[2]

[1] These investigators studied four proteins. They concluded that the ammonia liberated was approximately equal to the sum of the amide nitrogen and of half the arginine nitrogen. Under the conditions of hydrolysis which they employed, arginine actually yields only 35 to 42 per cent of its nitrogen as ammonia. Moreover, their method of determining amide nitrogen would tend to yield high values. The origin of significant amounts of ammonia from unidentified sources is not excluded by their observations.

[2] This is the name which has been suggested for the crystalline globulin isolated by Palmer (7) from the albumin fraction of cow's milk.

EXPERIMENTAL

Stock solutions of the proteins were prepared and their protein content calculated from determinations of dry weight (110°) and

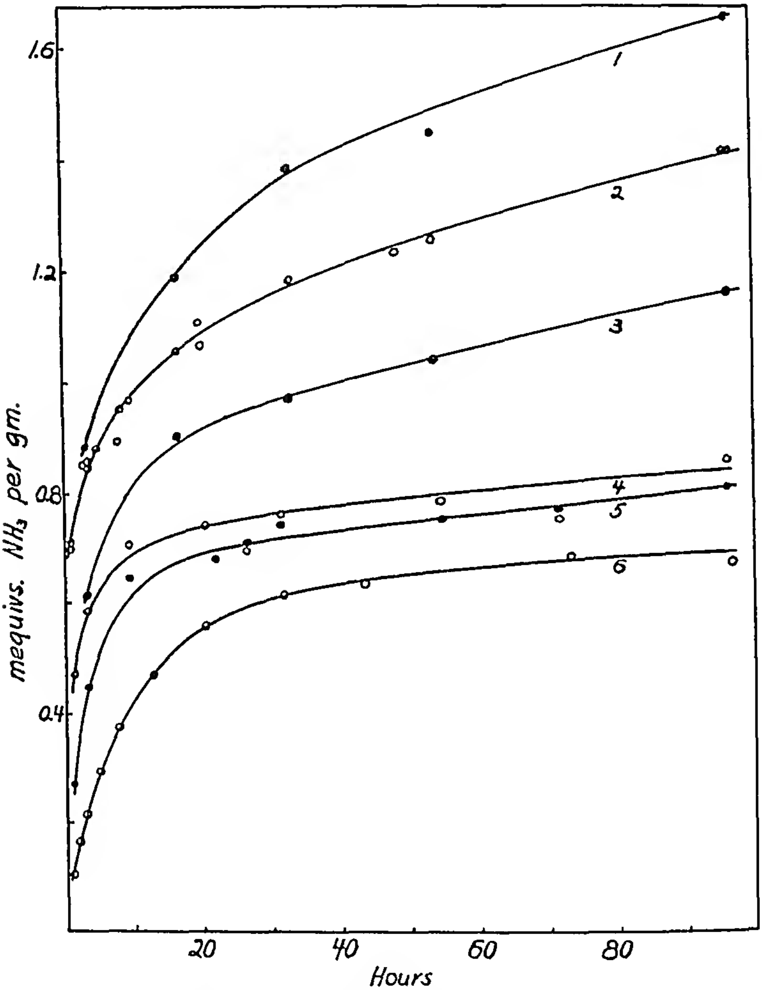

FIG. 1. Ammonia formation from egg albumin. Curve 1, 4.3 N NaOH at 68°; Curve 2, 1.4 N NaOH at 68°; Curve 3, 0.28 N NaOH at 68°; Curve 4, 4.3 N NaOH at 35°; Curve 5, 1.4 N NaOH at 35°; Curve 6, 1.2 N NaOH at 25°.

ash. Reaction mixtures were then prepared from measured amounts of these and of a standard solution of NaOH. Samples of each reaction mixture were sealed in a series of small Pyrex tubes which were then heated in a boiling water bath (100°) or a constant

temperature water bath (25°, 35°, or 68°). Tubes were removed at intervals, chilled in ice water, and the contents analyzed for ammonia by the method of Conway and Byrne (8).

The Conway and Byrne method requires a 2 to 3 hour distillation period at room temperature. During this time there is a slight liberation of ammonia from the amide groups. The short time observations at 25° and 35° are therefore of only qualitative significance. At higher temperatures the amide hydrolysis is sub-

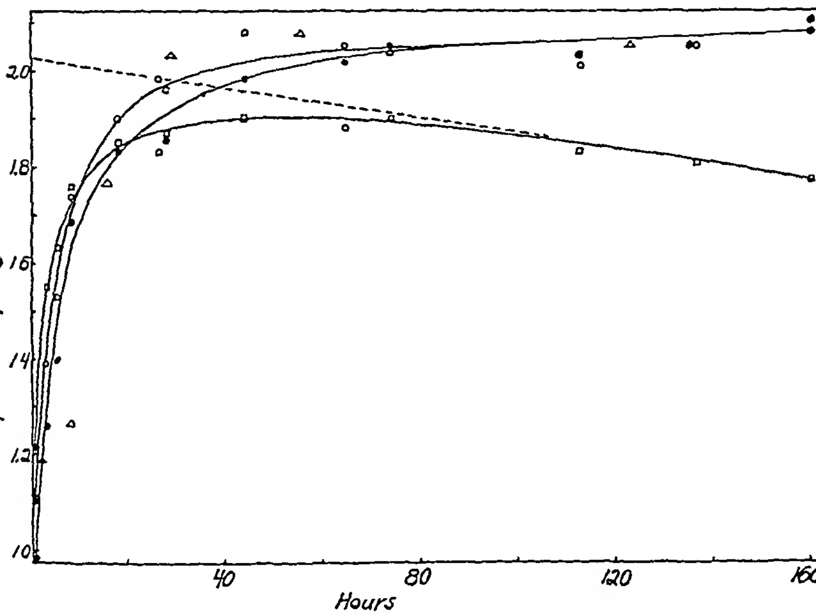

FIG. 2. Ammonia formation from egg albumin at 100°. ○ 1.4 N NaOH, ● 0.28 N NaOH, □ 4.3 N NaOH, △ 2.3 N $Ba(OH)_2$.

stantially complete even at the shortest reaction times employed.

In a few cases urea was estimated after the ammonia determination by titrating the reaction mixture with H_3PO_4 to pH 7.2, digesting with urease, and determining the ammonia formed.

Ammonia Formation from Egg Albumin—Representative data on egg albumin are shown graphically in Figs. 1 and 2. Inspection of the curves reveals the following characteristics.

At 35° the curves show an initial rapid formation of ammonia,

followed by a slow increase which continues throughout the period studied. Extrapolation of the slow rise back to zero time yields a value for the labile ammonia of 0.70 milliequivalent per gm. This is consistent with the maximum attained by the 25° curve and is identical with the value for amide nitrogen reported by Shore, Wilson, and Stueck (9). Their value was obtained by extrapolation of the time curves of ammonia formation in acid hydrolysis. It is interesting to note that M NaOH hydroylzes the amide groups at 35° at a rate comparable to that of M HCl at 100°.

Our results for the three proteins studied suggest that a simple and precise determination of amide nitrogen may be made in the following manner. Set up a series of Conway vessels containing a solution of the protein in M NaOH. Allow the spontaneous distillation to proceed at 25–35°. At chosen periods varying from 12 to 60 hours take one of the vessels and titrate the ammonia which has distilled. If the values at the successive reaction times are in close agreement, the mean may be taken as the amide nitrogen. If a progressive increase with time is observed, a linear extrapolation to zero time will give a close approximation to the true amide nitrogen.

At 68° the curves rise very rapidly to a value somewhat above the amide and then assume a fairly constant slope. With increasing concentration of NaOH this slope increases slightly and is displaced upward on the ordinate. The hydrolysis of arginine can be expected to contribute to the ammonia formed under these conditions (10).

At 100° the curves in 1.4 and 0.28 N NaOH rapidly reach a maximum of about 2.0 milliequivalents per gm. and show a slow rise above this figure with increasing time. In 4.3 N NaOH a maximum of only 1.8 to 1.9 milliequivalents is attained, following which there is a decrease in the amount of ammonia present. Extrapolation of the decreasing slope to 2.0 milliequivalents at zero time is not unreasonable (Fig. 2).

The maximum value of 2.0 milliequivalents per gm. is further confirmed by an experiment in 2.3 N $Ba(OH)_2$ at 100°, also shown in Fig. 2.

Ammonia Formation from Edestin and β-Lactoglobulin—More limited data have been obtained on the other two proteins, the graphs of which, in Figs. 3 and 4, show characteristics similar to

the curves for egg albumin. In Table I the values for the total alkali-labile ammonia as determined in 1.4 M NaOH at 100° and for the ammonia produced at 35° or 25° are compared with the analytical data on these proteins. The amide nitrogen estimated by extrapolation of the low temperature curves to zero time shows satisfactory agreement with the amide nitrogen determined by

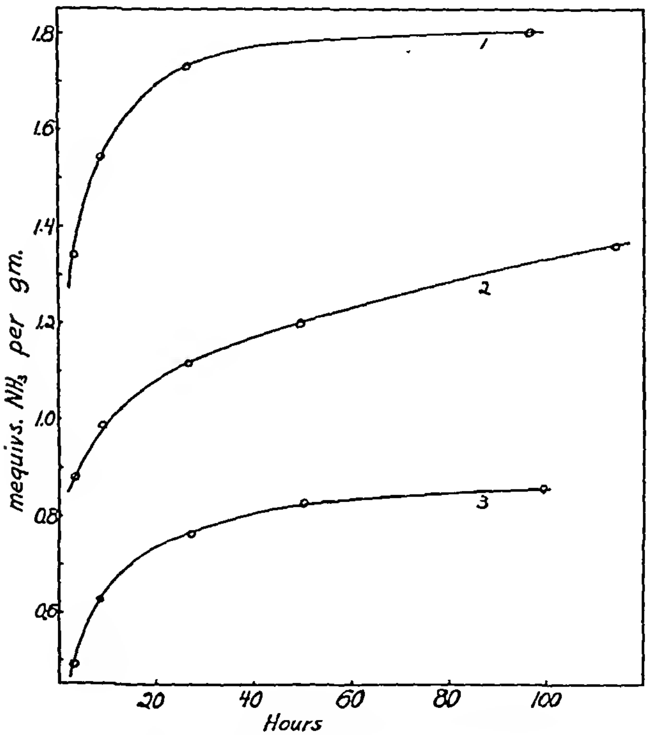

FIG. 3. Ammonia formation from β-lactoglobulin in 1.4 N NaOH. Curve 1, 100°; Curve 2, 68°; Curve 3, 35°.

other methods. In Column 6 are the values for the ammonia in excess of the sum of the amide and one-half of the arginine nitrogen.

The slow increase above the plateau in the curves at 100° in M NaOH probably represents contributions from such amino acids as cystine, histidine, and serine. Any ammonia that these acids may yield to the unknown fraction is neglected in the analysis of the rate

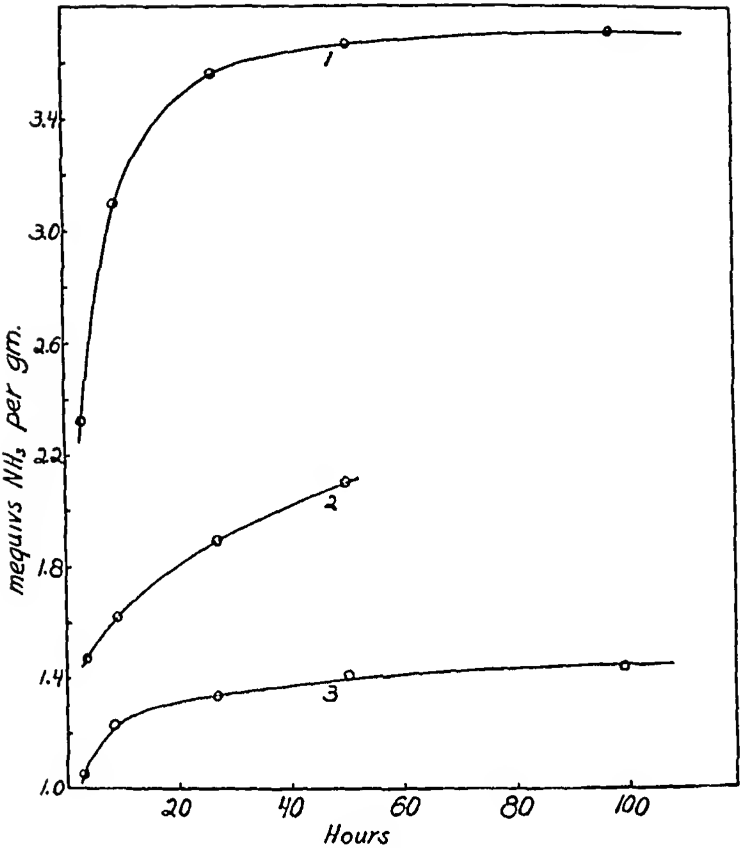

FIG. 4. Ammonia formation from edestin in 1.4 N NaOH. Curve 1, 100°; Curve 2, 68°; Curve 3, 35°.

TABLE I

Ammonia Formation from Three Proteins in Alkaline Solution

In Columns 1 and 2 are given data from the literature or from Appendix II. In Column 3 are the values for amide groups obtained by extrapolation of the experiments at 25° or 35°. Column 4 is calculated from Columns 1 and 3. The values obtained are subtracted from the total ammonia at 100° (Column 5) to give the ammonia from unidentified sources in Column 6.

The results are expressed in mM per gm.

	Arginine (1)	Amide groups (2)	Amide groups, NaOH experiments (3)	Amide + ½ arginine N (4)	Total alkali-labile NH_3 (5)	Unidentified NH_3 (6)
Egg albumin......	0.32 (11)*	0.70 (9)*	0.70	1.34	2.03	0.69
β-Lactoglobulin...	0.16†	0.77‡	0.78	1.10	1.77	0.67
Edestin..........	0.96 (11)*	1.37‡	1.34	3.25	3.71	0.45

* Bibliographic reference number.

† Vickery, H. B., personal communication.

‡ See Appendix II.

of ammonia formation which follows. An attempt to estimate the magnitude of such contribution is made later.

Rate of Formation of Unidentified Ammonia—An analysis of the rates of ammonia formation under various conditions of temperature and hydroxyl ion concentration suggests that two types of reaction are involved. Let us, first, correct the experimental curves for the contribution of amide and arginine groups. The total of the former can merely be subtracted at all points on the

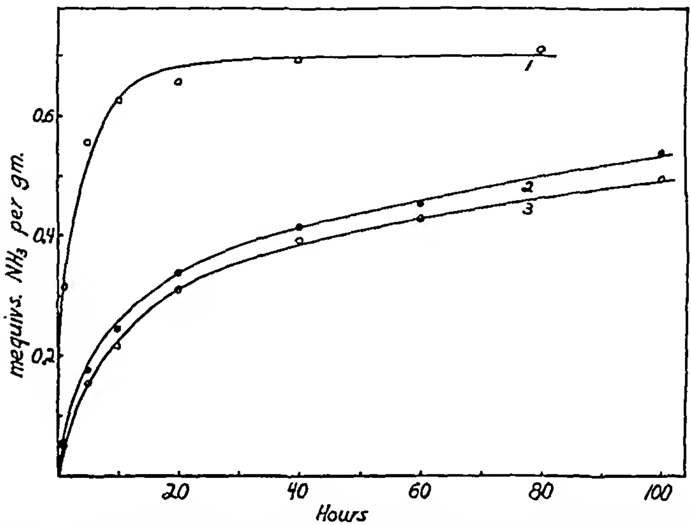

FIG. 5. Rate of formation of unidentified ammonia from egg albumin. The curves are theoretical plots obtained from the rate constants in Table II. Discrete points are the corrected values for the unidentified ammonia obtained as explained in the text. Curve 1, 1.4 N NaOH at 100°; Curve 2, 4.3 N NaOH at 68°; Curve 3, 1.4 N NaOH at 68°.

curves for 68° and 100°. Corrections for the ammonia formed from arginine were made on the basis outlined in Appendix I.[3]

[3] The chief primary products of the hydrolysis of arginine are urea and ornithine, most of the ammonia being formed secondarily from the urea. There should thus be an accumulation of urea in the reaction mixtures during the early stages of the hydrolysis. This was demonstrated by analysis in which urease was employed. The amounts of urea found agreed well with those calculated from the equation in Appendix I, assuming 0.32 mM of arginine per gm. of egg albumin.

The application of the corrections to the ammonia data obtained at 68° is shown in Figs. 5 and 6. The experimental values for the ammonia formed less the amide ammonia were plotted, and values were interpolated at intervals from a smooth curve drawn through the data. The arginine corrections calculated from the equation in Appendix I were subtracted and the values obtained were plotted as the discrete points on the lower curves of the graphs.

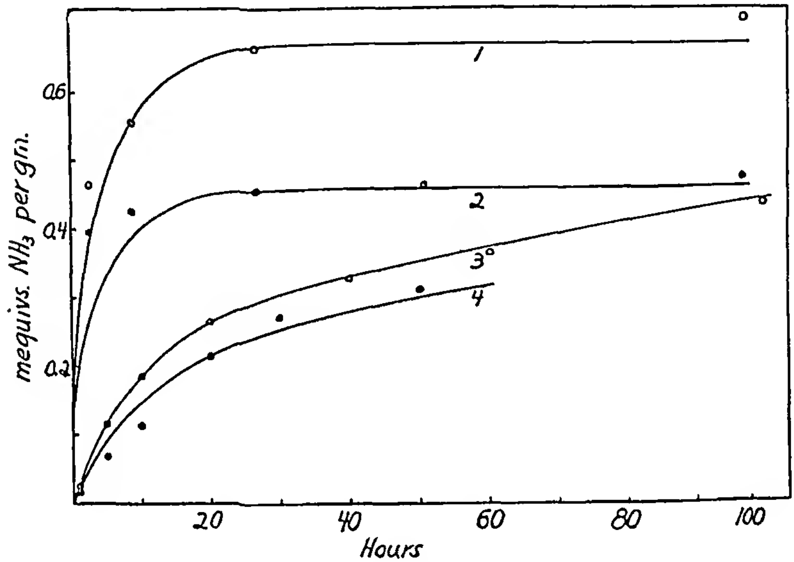

FIG. 6. Rate of formation of unidentified ammonia from β-lactoglobulin and edestin. The curves are theoretical plots obtained from the rate constants in Table II. Discrete points are the corrected values for the unidentified ammonia obtained as explained in the text. Curve 1, β-lactoglobulin in 1.4 N NaOH at 100°; Curve 2, edestin in 1.4 N NaOH at 100°; Curve 3, β-lactoglobulin in 1.4 N NaOH at 68°; Curve 4, edestin in 1.4 N NaOH at 68°.

Examination of the trend of these points shows a comparatively rapid initial reaction followed by a slower rise which is almost linear with respect to time. A curve of this type can be most simply accounted for as the sum of two independent first order reactions, having rates differing by a factor of about 10. The data have been treated on this assumption. Extrapolation of the linear portion of the curves to zero time should give an approximate value for the extent of the more rapid process. This value will be re-

ferred to as A_1. The extent of the slower process (A_2) will be taken as equal to the total unknown ammonia minus A_1.

First order constants, k_1 and k_2, for the two reactions and values for A_1 and A_2 have been derived to give the best fit with the data and are shown in Table II. The smooth curves in Figs. 5 and 6 are theoretical plots calculated from these constants.

This method of treatment was extended to the data at 100°. There are insufficient points on the rising portion of the curve to define the trend very accurately. The final corrected values for the ammonia are shown as the discrete points in the upper curves of the graphs (Figs. 5 and 6). The smooth curves through the data

TABLE II

Values for Extent of Two Processes, A_1 and A_2, and for Respective First Order Rate Constants Used in Plotting Theoretical Curves in Figs. 5 and 6

The rate constants are calculated in terms of common logs and reciprocal hours.

	A_1	A_2	k_1	k_2
Egg albumin	0.29	0 40		
68°, 4.3 N NaOH			0.06	0.004
68°, 1.4 " "			0.05	0.003
100°, 1.4 " "			0.8	0.07
β-Lactoglobulin	0.23	0.44		
68°, 1.4 N NaOH			0.05	0.003
100°, 1.4 " "			0.8	0 07
Edestin	0.17	0.29		
68°, 1.4 N NaOH.			0.05	0.005
100°, 1.4 " "			0.8	0.07

are theoretical plots obtained with the A_1 and A_2 values for each of the proteins derived from the data for 68° and with the average values of k_1 and k_2 which give the best fit with the data for all three proteins. The values used for A and k are shown in Table II. The derived curves adequately describe the experimental data. It should be noted that the errors in the case of edestin are relatively larger than for the other proteins because the amounts of amide and arginine present make the corrections large as compared with the unknown ammonia.

In fitting any individual curve it is not surprising that satisfactory values for A and k can be found, since variation can be

made in any of the four parameters which describe the curve (A_1, A_2, k_1, k_2). However, some confidence can be placed in this method of treating the data in view of the fact that constants of the same order of magnitude describe the curves for all of the proteins at the same NaOH concentration and that the same values for A_1 and A_2 give satisfactory results for egg albumin at different temperatures and NaOH concentrations.

Reaction A_1 can be followed to completion at 68° and the agreement is good enough to place some reliance in the statement that this is a first order reaction which proceeds to a definite maximum independently of the ammonia formation from other sources. It is quite possible that A_2 is a composite of several reactions, since it cannot be followed to completion under conditions which enable it to be considered separately from A_1.

Source of Unidentified Ammonia—If the ammonia is formed as a result of the decomposition of some specific amino acid, it should be possible to obtain it in the same quantity by acid hydrolysis of a protein followed by treatment of the hydrolysate with alkali under the conditions which defined the maximum ammonia production in the previous experiments. Data are given below on this type of experiment on egg albumin.

The maximum amount of ammonia obtained in excess of amide and arginine ammonia was only about 0.15 milliequivalent per gm. after 20 hours at 100° in M NaOH. Under these conditions the processes A_1 and A_2 should be substantially complete. This 0.15 milliequivalent per gm. is probably included in A_2 and may be composed of contributions from histidine, cystine, serine, and possibly other amino acids. No trace of the remaining 0.25 milliequivalent of A_2 or of the more labile 0.29 milliequivalent of A_1 could be found in these experiments.

The estimate of the unidentified ammonia should thus be reduced to 0.54 milliequivalent per gm. for egg albumin. This represents about 5 per cent of the total nitrogen in the protein. No data of this type were obtained on the other proteins studied, but the ammonia that cystine, histidine, and serine will yield cannot be much greater than for egg albumin. None of the proteins used contains much cystine, and the ammonia from histidine is small in any case. There are no satisfactory analytical data on the serine content of these proteins. However, the amounts which have been isolated

from most proteins are small, and under the conditions of these experiments it was found that the ammonia obtained from serine is equal to about 30 per cent of its nitrogen.

It can be concluded that the 0.54 milliequivalent of ammonia from egg albumin is not a result of the decomposition of amino acids as such. However, it probably comes chiefly from amino groups, since it is difficult to account for this amount of nitrogen from any other source. Hence, this ammonia must be referable to some structural feature of the protein itself, perhaps to certain peptide bonds which yield amino acids on acid hydrolysis and ammonia on treatment with alkali. There has appeared, after the completion of the manuscript of this paper, a note by Nicolet and Shinn (12) on the lability of serine and threonine when in peptide combination. It is probable that part of the unidentified ammonia arises from these sources. This question will be further investigated.

Experiment I—A sample of egg albumin was hydrolyzed for 24 hours with 20 per cent H_2SO_4 under a reflux. Aliquots of this hydrolysate were sealed in Pyrex tubes with a sufficient excess of NaOH to give a final concentration of 1.0 N. The tubes were heated in a boiling water bath and ammonia was determined on samples removed at intervals. The ammonia present in an unheated sample amounted to 0.813 milliequivalent per gm. or a little in excess of the amide ammonia. This was subtracted from the ammonia found in the heated samples. The results are summarized in Table III.

Experiment II—The basic amino acids were removed from a H_2SO_4 hydrolysate with phosphotungstic acid. The excess phosphotungstic acid was removed with $Ba(OH)_2$ and the aliquots of the filtrate were sealed in tubes with excess NaOH and were treated as above. The ammonia present in an unheated sample was 0.038 milliequivalent per gm. This has been subtracted from the results in Table III.

Appendix I. Derivation of Arginine Corrections—Corrections were made above for the amount of ammonia formed from arginine under various conditions. The equations from which these corrections were calculated were obtained in the following manner from a study of the kinetics of hydrolysis of arginine and urea (10).[4]

[4] References to equation numbers are from this paper.

The urea formation during the course of arginine hydrolysis can be described by combining Equations 3, 20, and 22. Since only alkaline hydrolyses are being considered, the k_2xy term in Equation 3 can be neglected. The equation

$$\frac{du}{dt} = \frac{k}{n} z - k_1 n$$

TABLE III

Ammonia Obtained by Treating H_2SO_4 Hydrolysate of Egg Albumin with Alkali

Experiment I shows the ammonia formed in excess of that present in the acid hydrolysate. Two other similar experiments showed 0.803 and 0.805 milliequivalents per gm. in 26 and 22 hours respectively. Experiment II shows the ammonia formed after removing the arginine. Another similar experiment yielded 0.18 milliequivalent per gm. in 21 hours.

After allowance was made for the arginine present in Experiment I, an average of 0.15 milliequivalent per gm. of ammonia in excess of the amide and arginine ammonia was titrated in 20 to 25 hours.

Experiment I		Experiment II	
Time	Ammonia	Time	Ammonia
hrs.	*m.eq. per gm.*	*hrs.*	*m.eq. per gm.*
3.1	0.313	19.4	0.148
15.9	0.461	98	0.177
23.7	0.759	162	0.191
40.5	0.794	282	0.214
63.6	0.806		
111.5	0.800		

was obtained. This was combined with Equation 23 and integrated to give

$$\frac{u}{a} = \frac{k}{n(k_1 - k)} (e^{-kt} - e^{-k_1t})$$

To obtain the corrections for the ammonia formed from arginine, Equations 4, 5, 25, and the above equation were combined, integrated, and solved for total ammonia (ΣNH_3). The k_2xy term in Equations 4 and 5 was neglected as before. The following equation was obtained.

$$\frac{\Sigma NH_3}{a} = 2 + Ae^{-kt} + Be^{-k_1t} + Ce^{-k_3t} + De^{-k_4t}$$

where

$$A = -\frac{k_1}{n(k_1 - k)}\frac{2k_3 - k}{k_3 - k} - \frac{1}{p}\left(\frac{2k_4 - k}{k_4 - k}\right)$$

$$B = \frac{k}{n(k_1 - k)}\frac{2k_3 - k_1}{k_3 - k_1}$$

$$C = -\frac{k_1 k}{n(k_3 - k_1)(k_3 - k)}$$

$$D = \frac{k}{p(k_4 - k)}$$

$$p = m + 1$$

$$n = \frac{m + 1}{m}$$

a = initial concentration of arginine

The constants have the same significance as in the previous paper (10).

The values for the constants required in the calculations are presented in Table IV. At 100° they were obtained by graphical interpolation from the data previously recorded. No systematic determinations on arginine hydrolysis were made at 100°. A single determination in N NaOH indicated that the value of k must be about 3.5. The reaction is thus so rapid that errors in k even of the order of 50 per cent will have relatively little effect on the calculations.

At 66° the values of the constants were obtained in the same way and were then corrected to 68° on the basis of approximate critical increments calculated between 66–100° for k_1, k_3, and k_4 and between 35–66° for k. Since the corrections are small, errors in the critical increments used are of little significance. It was assumed that m does not change with the temperature, although there is some indication that it is higher at 35° than at 66°. The calculations are here applied to proteins on the assumption that the rates of the several processes involved will not be affected by the incorporation of arginine in a peptide chain.

The only other simplification made was in neglecting reactions of OCN^- with amino acids, such as were described by Boon and Robson (13). By analogy with the formation of urea from NH_4^+

and OCN^- these would not be expected to occur in strongly alkaline solution.

$\Sigma NH_3/a$ was calculated for various time intervals by means of these constants. From these values, ΣNH_3 was obtained, assuming a to be 0.32 milliequivalent per gm. for egg albumin, 0.96 for edestin, and 0.16 for β-lactoglobulin (see Table I).

Table IV

Values for Velocity Constants Used in Calculating Ammonia Formed from Arginine during Hydrolysis of Proteins

$m = 5.5$.

Temperature, °C. / NaOH, N	100 / 1.4	68 / 1.4	68 / 4.3
k	3.5	0.240	0.262
k_1	0.234	0.00815	0.0525
k_3	0.170	0.0066	0.0066
k_4	0.0258	0.0013	0.0047

Table V

Ammonia Formation during Acid Hydrolysis of β-Lactoglobulin and Edestin

β-Lactoglobulin				Edestin			
0.25 M HCl at 85°		1 M HCl at 100°		0.25 M HCl at 85°		1 M HCl at 100°	
Time	NH_3	Time	NH_3	Time	NH_3	Time	NH_3
hrs.	*m.eq. per gm.*	*hrs.*	*m.eq. per gm.*	*hrs.*	*m.eq. per gm.*	*hrs.*	*m.eq. per gm.*
0.5	0.105	18	0.807	0.33	0.269	0.5	1.07
0.75	0.124	25	0.838	0.50	0.344	1.0	1.25
1.0	0.173	45	0.888	0.75	0.454	1.5	1.26
2.0	0.304	70	0.973	1.5	0.647	6.0	1.32
3.0	0.368	73	0.966	2.0	0.806	17.0	1.38
4.5	0.475	94	1.027	3.0	0.924	24.0	1.38
6.5	0.580	143	1.098	5.0	1.00	48.0	1.39
13.0	0.713			7.0	1.24	72.0	1.39
18.0	0.741			17.0	1.37		
24.0	0.754			24.0	1.37		
47.0	0.798						
49.0	0.798						

Appendix II. Amide Nitrogen of β-Lactoglobulin and Edestin—The methods used were those described by Shore, Wilson, and Stueck (9). These involve measurements of the time course of

ammonia production during acid hydrolysis. A summary of the results is given in Table V. The results with β-lactoglobulin show a rapid deamidation followed by a slow increase in ammonia formation at longer time intervals. In M HCl at 100° a linear extrapolation of this rise to zero time yields a value of 0.77 mM per gm. for the amide nitrogen of β-lactoglobulin. This value is confirmed by the experiment at the lower temperature. The secondary rise in the case of edestin is negligible. The results in several experiments, two of which are quoted here, are consistent with a value of 1.37 mM of amide per gm., substantially confirming values recorded in the literature (14).

SUMMARY

Data are presented on the rate and extent of the formation of ammonia at 25°, 35°, 68°, and 100° in various concentrations of NaOH from egg albumin, β-lactoglobulin, and edestin.

2. In all three proteins the amount of ammonia formed exceeded that due to the known sources which are amide groups and arginine.

3. The rate of formation of this excess ammonia can be most simply accounted for by assuming that it is produced from two independent sources by first order reactions.

4. Only a fraction of the unknown ammonia can be obtained by treating an acid hydrolysate of egg albumin with alkali. It is concluded that the ammonia is not formed by the decomposition of any amino acid as such, but it is referable to alkali-labile groups that exist in the protein.

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THE ALKALINE HYDROLYSIS OF EGG ALBUMIN

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Following the discovery of Kossel and Weiss (1) that many of the constituent amino acids are racemized when proteins are hydrolyzed by alkali, the use of alkaline hydrolysis in the analysis of proteins has been limited to the determination of amino acids which are unstable in strong acid. There is little information available as to the extent and completeness of hydrolysis as a function of $[OH^-]$ and temperature (2, 3). Vickery (4) showed that the initial reaction rate in the case of wheat gliadin was greater than that due to a comparable concentration of acid, and that it was greater with $Ba(OH)_2$ than with an equivalent concentration of NaOH. His observations did not extend to the late stages of hydrolysis. Now alkaline hydrolysates possess some apparent advantages to the analyst. The solutions are clear and there is no precipitation of humin. When $Ba(OH)_2$ is employed, it may be readily removed. Moreover, the racemization which occurs is not necessarily a disadvantage. We have, therefore, undertaken a detailed study of the course and completeness of the alkaline hydrolysis of egg albumin under a variety of conditions.

EXPERIMENTAL

Stock solutions of egg albumin were prepared from air-dried crystalline protein, obtained by the method of Kekwick and Cannan (5). The solutions were dialyzed, filtered, and standardized for protein concentration by dry weight and ash determinations.

Reaction mixtures were prepared by mixing measured quantities of stock protein solution and standard NaOH or solid $Ba(OH)_2$. Samples of these reaction mixtures were sealed in small test-tubes

and heated in a boiling water bath (100°), a constant temperature water bath at various temperatures, or in a boiling methyl alcohol bath (66°) under a reflux. Tubes were removed at intervals and immediately chilled in ice water. They were then opened and the ammonia which had formed was removed from an aliquot sample by distillation at room temperature in the units described by Conway and Byrne (6). This step was necessary as a preliminary to the amino determination, since ammonia will give a partial reaction with nitrous acid. When $Ba(OH)_2$ was used as the hydrolyzing reagent, it was necessary to dissolve the cooled reaction mixture in acetic acid before removing samples for ammonia distillation.

Samples of the ammonia-free solution were taken for amino nitrogen determination by the manometric method of Van Slyke (7). A reaction time of 12 minutes was adopted in order that the ϵ-amino group of lysine might be fully determined in addition to the α-amino groups. Free amino acids were determined in some cases by the ninhydrin reaction which has been put on a quantitative basis by Van Slyke and Dillon (8). The triketohydrindene hydrate used in the latter determination reacts only with a molecule containing a free carboxyl group and a free amino group (or imino as in proline) in the α position.

Comparable studies of the rate and course of the acid hydrolysis of egg albumin were made in order to serve as a basis for comparison. In most of these the ammonia was not removed prior to the amino determination.

Rate of Alkaline Hydrolysis—Most of the data obtained are summarized in the curves of Figs. 1 and 2. It is evident that the rate of liberation of free amino groups increases with the temperature and alkali concentration. There is a tendency for the curves to level off after an initial period of rapid reaction. This is very marked in all of the experiments at 100° and is also evident at 68° at the higher NaOH concentration. The curves in $Ba(OH)_2$ rise more rapidly than in NaOH and level off at higher values for the free amino groups.

A curious feature of the curves is the actual decrease in free amino groups with increasing time in 4.3 N NaOH at 100° (Fig. 2). The decrease is apparently due to a secondary reaction involving the free amino groups, such that the rate of this reaction exceeds

the rate of formation of new groups when about 7.5 milliequivalents per gm. have been formed. This secondary reaction was not found in the $Ba(OH)_2$ curves, although they were followed for similar periods of time. It is significant that it occurs under

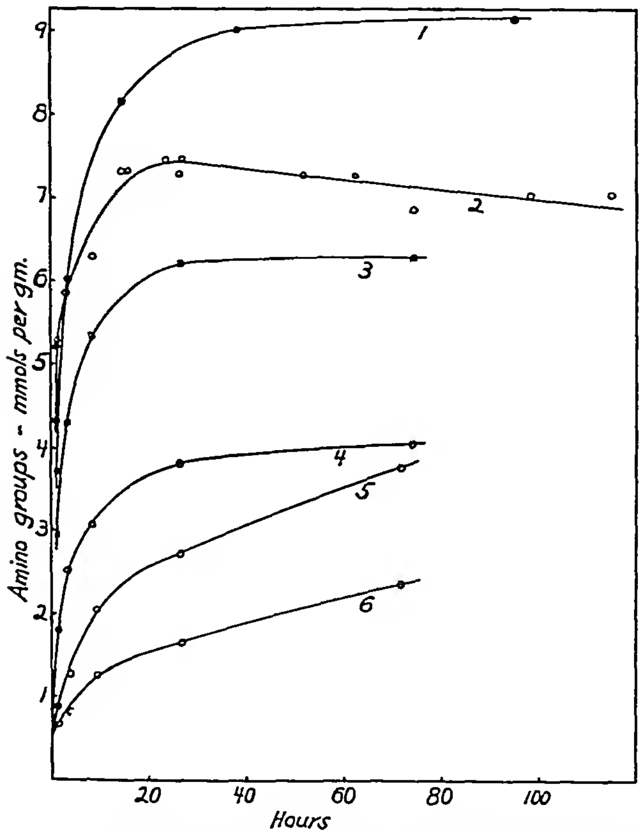

FIG. 1. Rate of formation of free amino groups during the hydrolysis of egg albumin. Curve 1, 20 per cent H_2SO_4 at 100°; Curve 2, 4.3 N NaOH at 100°; Curve 3, 1.4 N NaOH at 100°; Curve 4, 0.43 N NaOH at 100°; Curve 5, 4.3 N NaOH at 35°; Curve 6, 1.4 N NaOH at 35°.

conditions similar to those in which a secondary decrease in ammonia has been observed (9).

It will be noticed that none of the curves rises to the maximum value established by the experiment in 20 per cent H_2SO_4. How-

ever, in order to make a valid comparison of the acid and alkaline hydrolysates, it is necessary to apply certain corrections to the data. Since only α-amino groups enter into peptide bonds, consideration must be limited to these groups if a measure of the peptide splitting is desired. An estimate of the free α-amino

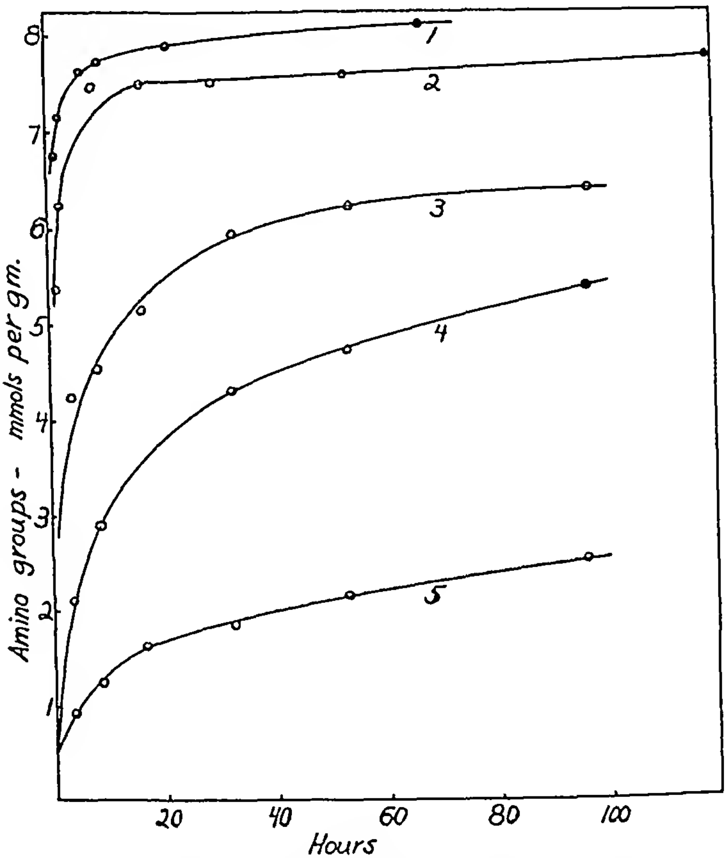

FIG. 2. Rate of formation of free amino groups during the hydrolysis of egg albumin. Curve 1, 3.7 N $Ba(OH)_2$ at 100°; Curve 2, 2.3 N $Ba(OH)_2$ at 100°; Curve 3, 4.3 N NaOH at 68°; Curve 4, 1.4 N NaOH at 68°; Curve 5, 0.28 N NaOH at 68°.

groups from the determinations of the total amino groups is attempted with the aid of the following corrections.

Corrections for Amino Data in 20 Per Cent H_2SO_4 Hydrolysis—In egg albumin the number of terminal amino groups of diamino acids amounts to 0.52 mM per gm. as measured by the nitrous acid

reaction with the unhydrolyzed protein. This value has been confirmed by titration of the protein in formaldehyde (Kekwick and Cannan (10)) and is used here, although it is definitely higher than the published analytical figures for lysine in egg albumin. Consequently, 0.52 mM per gm. has been subtracted from the free amino groups.

Proline and hydroxyproline do not react with nitrous acid, but they presumably enter into peptide bonds. The analytical value for the sum of these acids in egg albumin is 0.33 mM per gm. (11). This has been added to the value for free amino groups.

The ammonia formed during the hydrolysis was not removed in this case. It will introduce an error because of its reaction with nitrous acid, amounting to 67 per cent of the theoretical yield in 12 minutes. It has been assumed that the ammonia present arises from the hydrolysis of the 0.70 mM of amide groups (12). 0.47 mM per gm. has therefore been subtracted.

Corrections for Amino Data on Alkaline Hydrolysis—In alkaline solution arginine is hydrolyzed to ammonia and ornithine. The latter amino acid contains a terminal amino group which will react with nitrous acid. The formation of ornithine is substantially complete at 100° in strong alkali by the time the free amino curves have reached their maxima (13). Since there is 0.32 mM of arginine (14), the correction for diamino acids must be increased to 0.84 mM. This value has been subtracted from the amino determination.

The proline correction of 0.33 mM per gm. has been added.

The free ammonia was removed from the reaction mixtures before amino determinations were made. However, there is 0.69 mM per gm. of ammonia formed from a source other than amide groups, but not specifically known (9). This ammonia probably arises from α-amino groups, as it is difficult to suggest any other source. A correction of 0.69 mM, which will be referred to as the unidentified ammonia correction, has therefore been added to the data, although there may be some question as to the validity of so doing.

Comparison of Extent of Hydrolysis by Acid and Alkali—These corrections, applied to the maximum value attained in various hydrolysates, are presented in Table I. There is good agreement between the values obtained in H_2SO_4 and on HCl hydrolysis.

The maximum value for the 4.3 N NaOH hydrolysis is 0.69 mM per gm. below the acid value, even including the unidentified am-

TABLE I

Corrected Maximum Values for Amino and Ninhydrin Determinations in Various Hydrolysates

Explanations of the corrections will be found in the text. The data are calculated in mM per gm. of protein and the totals are also given in groups per mole of protein, assuming the molecular weight of egg albumin to be 34,500.

Acid hydrolysates	20 per cent H_2SO_4	Groups per mole	20 per cent HCl	Groups per mole
	mM per gm.		*mM per gm.*	
Observed amino N	9.01		8.64	
Less amide ammonia correction	0.47		*	
" diamino correction	0.52		0.52	
Plus proline "	0.33		0.33	
Total α-amino N	8.35	288	8.45	291
Observed ninhydrin			8.70	
Less aspartic acid correction			0.52	
Total free amino acids			8.18	282
Alkaline hydrolysates	**4.3 N NaOH**	**Groups per mole**	**3.7 N $Ba(OH)_2$**	**Groups per mole**
Observed amino N	7.48		8.12	
Less diamino correction	0.84		0.84	
Plus proline "	0.33		0.33	
" unidentified ammonia correction	0.69		0.69	
Total α-amino N	7.66	264	8.30	286
Observed ninhydrin	6.46		6.84	
Less aspartic acid correction	0.52		0.52	
Plus unidentified ammonia correction	0.69		0.69	
Total free amino acids	6.63	229	7.01	242

* In this case ammonia was removed before the amino determination was made.

monia correction. The more rapid $Ba(OH)_2$ hydrolysis shows good agreement with the acid value.

Some additional information can be obtained from the ninhydrin determinations. These have not been plotted graphically, but, in general, they rise more slowly than the number of free amino

groups and do not attain the same maximum values. In order to obtain the number of free amino acids, these figures must be corrected for aspartic acid, which yields twice the quantity of CO_2 given by any of the other amino acids. 0.52 mM per gm.[1] is accepted as the analytical value for aspartic acid and is subtracted from the experimental total for the ninhydrin reaction. The unknown ammonia correction is also applied in the case of the alkaline hydrolysates. These data are shown in Table I with the corresponding amino data.

In a completely hydrolyzed protein α-amino groups should occur only in free amino acids, and the two methods of determination should give the same value. The agreement for the HCl hydrolysate is satisfactory, the higher value for the amino groups probably being due to the unavoidable secondary reactions in the amino analysis. For the NaOH and $Ba(OH)_2$ hydrolysates there are deficits of 1.03 and 1.29 mM, respectively, in the number of free amino acids found.

The low value for amino groups in 4.3 N NaOH, as compared with the acid totals, is probably referable to the secondary reaction, previously mentioned, which decreases the free amino groups. A very rough correction for this consists of a straight line extrapolation of the decreasing portion of the curve in Fig. 1 to zero time. This raises the maximum value by about 0.5 mM per gm. to give a corrected figure of about 8.16 mM. No explanation can be offered for the deficit in free amino acids for the alkaline hydrolysates.

The results reported above suggest that the following considerations should govern the use of alkaline hydrolysates for analytical work on amino acids. (1) $Ba(OH)_2$ should be used in preference to NaOH. A concentration of at least 3 to 4 N is required to produce maximum hydrolysis. (2) The hydrolysis should be carried out at 100° or at the boiling point of the solution for probably not more than 10 hours. This gives almost the maximum yield of amino groups, avoids any extensive secondary reactions, and keeps to a minimum contamination of the hydrolysate with material dissolved from the wall of the glass vessel by the alkali. This can also be kept low by using a *new* Pyrex vessel for each

[1] Chibnall A. C. personal communication.

hydrolysis. Even shorter periods of hydrolysis might be usefully employed. (3) Caution should be exercised in accepting results obtained by alkaline hydrolysis *until it is established that the* amino acid in question does not contribute to the unidentified ammonia. The failure of the free amino acids to equal the amino groups at the end of the hydrolysis also raises some question as to the advisability of employing alkaline hydrolysis for analytical work.

Formation of Free Amino Acids during Hydrolysis of Protein—While at the completion of hydrolysis of a protein one expects the number of amino groups to equal the number of free amino acids, at any point corresponding to partial hydrolysis the former should be in excess. This is evident, since the hydrolysis of any peptide bond produces an amino group but a free amino acid is formed in general only by the hydrolysis of two adjacent bonds.

The theoretical relationship between the number of free amino acids and the number of bonds hydrolyzed throughout the course of the reaction has been examined on the basis of the following assumption. (1) A molecule of egg albumin has a molecular weight of 34,500 and consists of 288 amino acids[2] joined in a single chain by 287 peptide bonds. (2) The intrinsic rate of hydrolysis of all of the bonds is the same and is independent of the length of the chain, the position of the bond in the chain, and the particular amino acids forming the bond.

On these assumptions the rate of hydrolysis of bonds should follow the first order law, $kt = \log (287)/(287 - x)$, where x is the number of bonds hydrolyzed. The bonds will be hydrolyzed according to a random distribution, and the number of free amino acids formed when any given number of bonds has been hydrolyzed should equal a calculable expectancy.

Calculation of Expectancy of Free Amino Acids—A straight chain polypeptide, containing n peptide bonds, numbered 1 to n for convenience, and $n + 1$ amino acids is assumed. The problem is to find the expectancy of free amino acids when r bonds have been hydrolyzed. A random disposition of the hydrolyzed bonds within the molecule is assumed.

[2] 288 is used for the total number of acids because it agrees well with the maximum value for the α-amino determination in acid hydrolysates, and because it is the number of amino acid residues per mole assumed by Bergmann and Niemann (15).

Free amino acids will be formed if one or both of the end bonds (1 and n) are hydrolyzed, or if two or more consecutive bonds within the molecule are hydrolyzed. If m consecutive bonds are hydrolyzed, $m - 1$ free amino acids will be formed. Any given way of distributing the r hydrolyzed bonds among the n total bonds will be called a combination. Each combination must be given a weight, w, equal to the number of free amino acids produced when that combination is realized. The total number of possible combinations when r bonds are hydrolyzed will be $n!/(r!(n - r)!) = t$. The expectancy, E, may now be defined as the summation of all the possible combinations which produce at least one free amino acid, each multiplied by the weight, w, characteristic of that combination, and the whole divided by the total number of possible combinations, t.

The expectancy when r bonds are hydrolyzed (E_r) can be divided into three parts.

1. Expectancy from hydrolysis of both end bonds (E_{r_1}), *i.e.* 1 and n.

The number of combinations in which both 1 and n appear =

$$\frac{(n-2)!}{(r-2)!\,(n-r)!} = p$$

$w = 2$ for each of these combinations, since one free amino acid is formed for the splitting of each bond.

$$E_{r_1} = \frac{2p}{t} = \frac{2r(r-1)}{n(n-1)}$$

2. Expectancy from hydrolysis of a single end bond (E_{r_2}). The number of combinations in which either 1 or n (or both) appears =

$$\frac{(n-1)!}{(r-1)!\,(n-r)!} = m$$

$m - p = q =$ the number of combinations in which only one of the terminal bonds appears. This must be multiplied by 2 because there are two terminal bonds.

$$w = 1; \qquad E_{r_2} = \frac{2q}{t} = \frac{2r}{n}\left(\frac{n-r}{n-1}\right)$$

3. Expectancy from hydrolysis of consecutive bonds within the molecule (E_{r_3}): (*a*) number of combinations of two consecutive bonds when only 2 bonds are hydrolyzed $= n - 1 = a$; (*b*) number of combinations of the remaining $(r - 2)$ bonds $= (n - 2)!/(r - 2)!(n - r)! = b$; (*c*) total combinations containing two or more consecutive bonds, $a \cdot b = c$. The quantity c will contain combinations counted several times. The number of times a combination will be counted will be 1 less than the number of consecutive bonds that it contains. This factor is equal to the number of free amino acids produced when that combination is realized. Each combination is therefore properly weighted as calculated in (*c*).

$$E_{r3} = \frac{c}{t} = \frac{r(r-1)}{n}$$

4. The total expectancy of free acids is the sum of these three components

$$E_r = E_{r1} + E_{r2} + E_{r3} = \frac{r(r+1)}{n} \qquad (1)^3$$

The calculations have been applied to a hydrolysis of egg albumin in 1.0 N HCl at the boiling point of the solution. The data are recorded in Table II where the corrected values are tabulated alongside the experimental figures. The corrections were made in the same manner as previously, except that when hydrolysis is only partial it is unreasonable to apply full corrections for proline and aspartic acid. The corrections have been obtained by adding or subtracting a percentage of the amino acid equal to the percentage of free amino groups or the percentage of free amino acids for the nitrous acid and ninhydrin reactions, respectively. The corrected amino determination has been taken as a direct measure of the number of bonds hydrolyzed.

The amino data give a reasonably good first order constant, except for the point at 17 hours. The values for this constant at each point are also shown in Table II. In Fig. 3 the amino data

[3] After these equations were derived, it was found that Montroll and Simha (16) had worked out a more general treatment of a similar problem. The equations of these authors yield substantially the same numerical results when applied to the special case considered above.

are plotted and a smooth curve drawn through them. This curve is a plot of r as a function of time of hydrolysis. The values for the expectancy of free amino acids were plotted by calculating E_r from Equation 1 for values of r interpolated from the curve at various time intervals. The curve obtained is the lower curve in Fig. 3. The experimental values for the free amino acids are seen

TABLE II

Hydrolysis of Egg Albumin by 1.0 N HCl under Reflux

See the text for an explanation of the corrections. The diamino correction is reduced to 12 because the reaction time in the amino determination was 3 minutes in this experiment. 67 per cent of the ϵ-NH_2 of lysine will react in this time. The amide ammonia correction was estimated from the data of Shore, Wilson, and Stueck (12), assuming a 25 per cent reaction of ammonia with nitrous acid in 3 minutes. All data are recorded as groups per mole of 34,500 gm.

Time	Amino	Amide NH_3 correction	Proline correction	Corrected value*	k common logs hrs^{-1}	Ninhydrin	Aspartic acid correction	Corrected value
hrs								
0.25	27.2	0.04	0.01	15.2	0.092	2.4	0.1	2.3
0.50	36.2	0.07	0.5	24.3	0.076	6.4	0.4	6.0
0.75	43.8	1.0	0.8	31.6	0.067	8.8	0.6	8.2
1.00	58.6	1.3	1.3	46.6	0.078	13.4	0.8	12.6
1.50	82.4	1.9	2.4	70.9	0.083	19.4	1.2	18.2
2.00	100.6	2.4	3.2	90.4	0.082	29.4	1.8	27.6
3.00	129.8	3.2	4.4	120	0.078	51.0	3.2	47.8
4.00	146.6	3.8	5.1	137	0.071	68.6	4.3	64.3
6.00	180.2	4.7	6.5	171	0.066	103.8	6.5	97.3
8.00	197	5.0	7.2	188	0.058	128.0	8.0	120.0
17.00	223	6.9	8.3	214	0.035	168.6	10.2	158.4

* The diamino correction of twelve groups per mole has been subtracted in each case to obtain the values in this column.

to be in excellent agreement with the calculations, except in the very early stages of the hydrolysis in which the experimental error is large.

This agreement may seem surprising in view of the artificial nature of the assumptions made. However, there could be considerable variation in the assumptions with only small effect on the calculations. Actually, it is only necessary to agree that the

phenomenon being measured here is the hydrolysis of peptide bonds, and that the intrinsic rate of hydrolysis of different bonds by acid does not show a very wide distribution about the mean. The agreement in the above experiment can perhaps be taken as a demonstration of the latter.

The effect on the expectancy calculation of the assumption made in regard to the molecular weight, *i.e.* $n = 287$, can be seen by

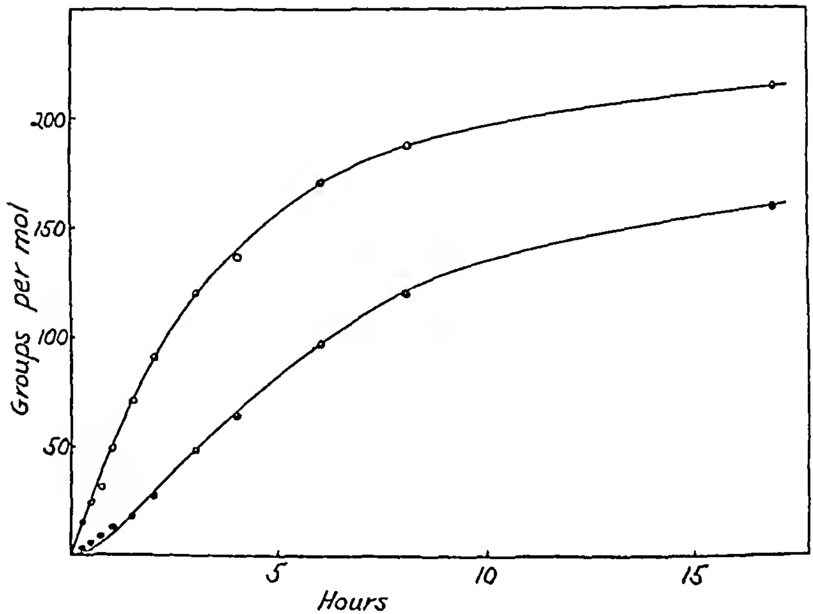

FIG. 3. Hydrolysis of egg albumin by 1.0 N HCl at 100°. Upper curve, increase in free amino groups; lower curve, formation of free amino acids. The points are corrected experimental data and the smooth curve is obtained as explained in the text.

calculating the fraction of free amino acids produced, $(E_r)/(n + 1)$, as a function of the fraction of bonds hydrolyzed (r/n). $(E_r)/(n + 1)$ will be independent of the molecular weight if it is a function of r/n only. From Equation 1 it can be seen that

$$\frac{E_r}{n + 1} = \frac{r(r + 1)}{n(n + 1)}$$

This will vary with n only in so far as $(r + 1)/(n + 1)$ differs from r/n. This difference will be appreciable only in the very early

stages of the hydrolysis. The same consideration shows that there would be only a small effect on the calculation if the molecule consists of several chains of peptides rather than a single one.

The same calculations when applied to the data obtained on alkaline hydrolysis yield different results. Plots similar to that

TABLE III

Hydrolysis of Egg Albumin by $Ba(OH)_2$ and NaOH

See the text for explanation of the corrections. All data are recorded as groups per mole of 34,500 gm.

Time	Amino N	Diamino correction	Proline correction	Unidentified NH_3 correction	Corrected values	Ninhydrin	Aspartic acid correction	Unidentified NH_3 correction	Corrected values
3.7 N $Ba(OH)_2$ at 100°									
hrs.									
1.03	233	27	9	10	225	174	11	6	169
2.00	246	28	10	20	246	193	12	13	194
6.09	264	28	10	21	267	202	13	15	204
9.42	267	29	11	23	272	232	15	18	235
21.4	273	29	12	24	280	229	15	18	232
66.5	280	29	12	24	287	236	15	20	241
1.45 N NaOH at 66°									
1.00	44.6	19.0	1.1	1.2	27.9	8.9	0.6	0.03	8.4
2.00	57.4	21.0	1.5	2.5	40.4	15.4	0.9	0.1	14.5
4.10	80.8	22.0	2.4	4.6	65.8	30.5	1.9	0.5	29.1
8.35	102.0	26.0	3.1	6.8	85.9	43.8	2.7	1.0	42.1
16.2	122.8	28.5	3.8	9.6	107.7	54.6	3.4	1.8	53.0
32.3	154.9	29	5.2	12.8	143.9	92.0	5.8	4.1	90.3
65.7	189	29	6.6	15.1	181.7	119.3	7.4	7.5	119.4
121.7	212	29	7.6	18.0	208.6	160.2	10.1	10.0	160.1
256.4	233	29	8.4	21.6	234.2	175	11.0	13.1	177.1
381.4	240	29	8.8	23.0	242.8	188	11.7	15.0	191.0

made on the HCl experiment are shown in Fig. 4 for 1.45 N NaOH at 66° and in Fig. 5 for 3.7 N $Ba(OH)_2$ at 100°. The data taken from Table III have been corrected as before, except that the fractional corrections for arginine and unknown ammonia have been made on the basis of the results previously obtained under similar experimental conditions (9, 13).

In neither of the cases do the amino determinations follow a first order equation. The reaction is very rapid initially and falls off as the hydrolysis proceeds. For the slower reaction in Fig. 4 the experimental values for free amino acids exceed the expectancy up to a value of 170 to 180 and then fall below it. In the more

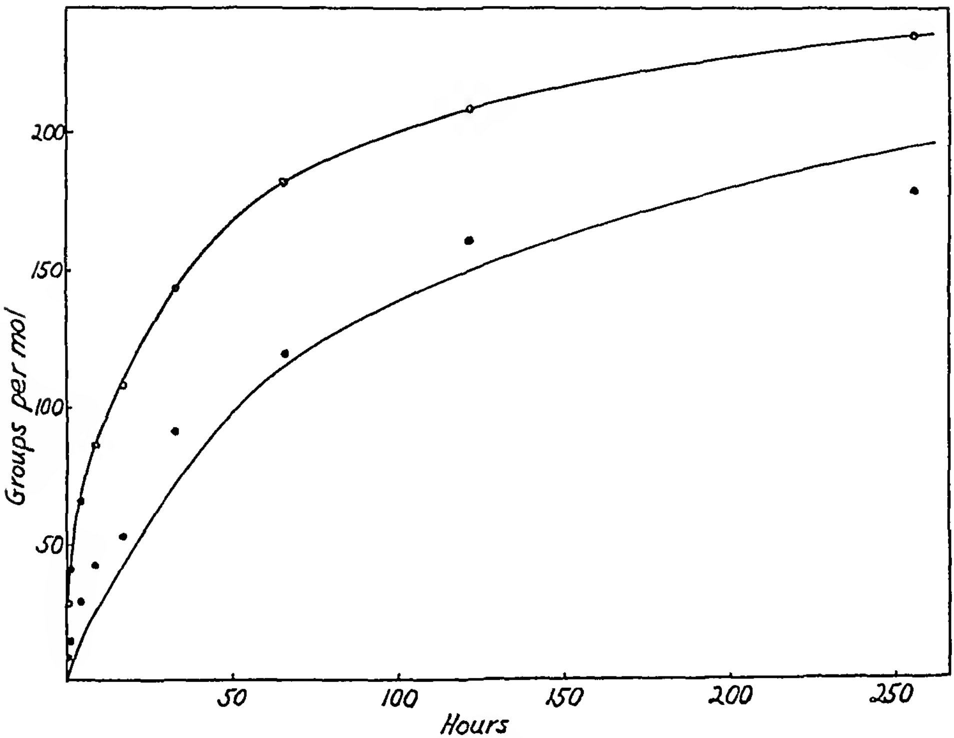

FIG. 4. Hydrolysis of egg albumin by 1.45 N NaOH at 66°. Upper curve, increase in free amino groups; lower curve, formation of free amino acids. The points are corrected experimental data and the smooth curve is obtained as explained in the text.

rapid $Ba(OH)_2$ hydrolysis, most of the points fall below the expectancy, but since these are chiefly above 180 they bear out the NaOH results.

The failure to come up to the expectancy toward the end of the reaction is a reflection of the failure of the free amino acids to rise to the α-amino group maximum as recorded in Table I. The initial rise above the expectancy and the initial rapid hydrolysis of

bonds possibly indicate that certain bonds or certain groups of bonds are more labile to alkali than the general average. The data for the other experiments in NaOH give similar results when plotted in the same fashion.

There is no essential difference in the curves if they are plotted without the unidentified ammonia correction.

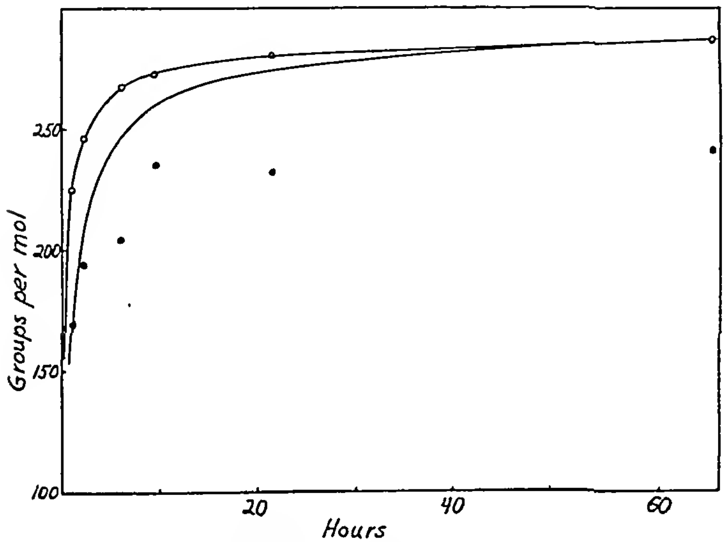

FIG. 5. Hydrolysis of egg albumin by 3.7 N $Ba(OH)_2$ at 100°. Upper curve, increase in free amino groups; lower curve, formation of free amino acids. The points are corrected experimental data and the smooth curve is obtained as explained in the text.

The author is indebted to Professor R. K. Cannan for his advice and criticism during the course of this work.

SUMMARY

1. Data are presented on the rate of the alkaline hydrolysis of egg albumin under various conditions of temperature and hydroxyl ion concentration, and on the completeness of the reaction relative to acid hydrolysis. $Ba(OH)_2$ was found to hydrolyze the egg albumin at a greater rate and more completely than NaOH.

2. A secondary reaction leading to a decrease in free amino groups with increasing time of hydrolysis was observed in 4.3 N NaOH at 100°.

3. Determinations by the ninhydrin-CO_2 method of the formation of free amino acids during the course of alkaline hydrolysis show that these do not rise to the value anticipated from amino determinations made by the nitrous acid method.

4. A theory is presented to account for the rate of formation of free amino acids as a function of the number of peptide bonds hydrolyzed. The theory accounts for the course of the hydrolysis by acid, but fails with alkaline hydrolysis.

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THE EXCRETION OF ESTROGENS IN THE BILE AND URINE AFTER THE ADMINISTRATION OF ESTRONE*

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The rôle of the bile as a pathway for the excretion of the metabolic products of the various estrogens has received scant attention. Two patents report the presence of estrogens in the bile (1, 2). Gsell-Busse (3) accomplished the extraction of estrogens from the bile of various species. The most extensive report is that of Stamler (4) who injected "folliculine" intravenously into dogs and followed the time curve of excretion in both the urine and the bile. He reported that urinary excretion was completed in 21 to 30 hours, whereas the bile ceased to contain estrogen 4 hours after the injection. The urinary excretion averaged 13 per cent of the amount injected, and the bile contained "not less than 13 per cent." He noted "no essential difference" when the hormone was given subcutaneously. Apparently he did not study the two paths of excretion simultaneously in the same dog, nor did he attempt to distinguish the form in which the active substance was excreted. Dingemanse and Tyslowitz (5) recovered estrogenic substance from the bile of dogs which had been treated with diethylstilbestrol. Previous to these studies Dohrn and Faure (6) had reported the presence of estrogens in the feces of gravid women.

The recovery in the urine of only a small part of the hormone after the injection of an estrogen has been established by the work of Zondek (7), Smith and Smith (8), Westerfeld and Doisy (9),

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† A portion of the material presented herein is from a thesis submitted to the Graduate School of the University of Colorado by Frances S. McKee in partial fulfilment of the requirements for the degree of Master of Science.

Pincus and Zahl (10), and Dingemanse and Tyslowitz (5). A part of the responsibility for this evident destruction has been placed upon the liver by the work of Zondek (7), Israel, Meranze, and Johnston (11), Heller, Heller, and Sevringhaus (12), Heller (13), Talbot (14), and Biskind and Mark (15).

Heller's research (13), which is not entirely comparable to the other reports because it was carried out by *in vitro* methods, demonstrated the transformation of estrone to a more active substance, probably α-estradiol, by a number of tissues. The uterus was the most active tissue in this reaction. The liver did not accomplish such a transformation, but did inactivate completely both estrone and α-estradiol. When the reaction mixture of liver and estrone was treated with sodium cyanide, however, α-estradiol was formed. These findings led the author to the conclusion that the liver converted estrone to α-estradiol and then rapidly inactivated the latter compound.

Ketonic estrogens were found in the urine after the injection of α-estradiol or one of its conjugates by Westerfeld and Doisy (9), Fish and Dorfman (16), and Heard and Hoffman (17). Non-ketonic estrogens were present in the urine of the dogs treated with estrone in the experiments of Dingemanse and Tyslowitz (5). These findings show that the metabolic processes are capable either of the transformation of estrone to non-ketones or of the conversion of α-estradiol to estrone.

The bile is a medium from which valuable information may be gained concerning these metabolic reactions. To be sure, it does not necessarily follow that any substance found in the bile has undergone a reaction by the liver. Such substances may represent excretory products which have not been changed by passage through the liver. The evidence is clear, however, that the liver is intimately concerned in the metabolism of the estrogens, and the bile seems a likely source of material with which to study these reactions.

The following discussion is a report of experiments in which the excretion of estrogens in the bile and in the urine was studied simultaneously after the administration of estrone to female dogs.

Methods

Bile fistulas were prepared according to the method of Virtue and Doster-Virtue (18). The animals were maintained on a

complete stock diet with supplements of cod liver oil and yeast tablets. Except during the experimental periods, the collected bile was fed to the dog daily.

The urine and bile specimens were collected over a period of 72 hours with the usual precautions to insure quantitative collection for metabolism studies. The urine was preserved with hydrochloric acid during the collection. The bile specimens, which were collected daily during the experiment, were also preserved with hydrochloric acid and were kept in the refrigerator until the conclusion of the period. At the beginning of each experiment 1.0 mg. (10,000 I.U.) of estrone in oil was injected subcutaneously.

Extraction and fractionation of the bile and urine, respectively, were accomplished by the method of Dingemanse, Borchardt, and Laqueur (19) as modified by Callow, Callow, Emmens, and Stroud (20). The method was not varied except in the case of the extraction of the benzene solution of bile extract with aqueous sodium bicarbonate, in which step three or four washings were used instead of the two prescribed for urine extracts. The greater concentration of protein in bile posed the question whether the extraction of steroids would be complete in its presence. A control experiment was done in which an extraction was carried out in the usual way after the addition of 60 γ of estrone to 450 cc. of bile. Recovery was 100 per cent.

The ketonic estrogens were separated from the non-ketones with the Girard-Sandulesco Reagent T (21). Its effectiveness with bile was determined by separation of ketones from non-ketones after the addition of 60 γ of estrone to the phenolic fraction. 80 per cent of the estrone was recovered in the ketonic fraction. Westerfeld, Thayer, MacCorquodale, and Doisy (22) demonstrated the adequacy of this method with minute amounts of estrogens and reported the recovery of the ketonic compound to be from 70 to 100 per cent.

The fractionation of the non-ketones was accomplished by the method of Mather (23).[1] Huffman, Thayer, and Doisy (24) confirmed the efficiency of this method, which utilizes the difference in solubility of α-estradiol and estriol in 0.3 M sodium carbonate.

The vaginal smear method was used for the assay of the estrogenic fractions of the bile and urine, respectively, and the assays

[1] Mather, A., personal communication.

were performed on ovariectomized rats by an adaptation of the single subcutaneous injection technique of Gustavson, Mason, Hays, Wood, and D'Amour (25). The material was dissolved in oil for injection. The rats were standardized previous to the assays with crystalline estrone.

At least one control experiment was run on each dog. This was similar in every respect to the injection experiment except that the dog received no estrone.

Results

Control Experiments—The control experiments gave no evidence of the presence of estrogens in either the bile or the urine. When the entire phenolic fraction was injected into a single assay rat, there was no vaginal response. The ability of the rat to respond was demonstrated by its positive response in later experiments with active extracts. Therefore, we conclude that there was less than 1 rat unit (12 I.U.) in these extracts.

Total Excretion in Bile—The results of all experiments are recorded in Table I. In eight of the nine experiments, *i.e.* with the exception of Experiment 2, the minimum biliary excretion was 5.0 per cent and the maximum was 8.0 per cent of the injected estrone. In Experiment 2, Dog 1, the excretion was only 1.3 per cent. This animal had become emaciated before the experiment was begun, and it is possible that the function of the liver had become sufficiently impaired to affect the excretion of estrogen. Experiment 8 was the only instance in which the estrogen concentration in the bile was greater than that in the urine.

Total Excretion in Urine—Again except in Experiment 2, eight of the nine experiments showed a minimum urinary excretion of 6.4 per cent and a maximum of 9.7 per cent of the amount injected. In Experiment 2 the urinary excretion was 13.5 per cent, an amount which was significantly above the average of the other experiments at the same time that the biliary excretion was definitely below the average.

Excretion of Ketones and Non-Ketones—Separation of the ketones and non-ketones of five of the bile extracts showed the non-ketonic fraction to be from 92 to 107 per cent of the total estrogen. These values indicate that all of the estrogen excreted in the bile was in the non-ketonic form.

On the other hand, similar treatment of three of the urinary extracts gave values of the non-ketonic fraction of 61.8, 78.1, and 80.9 per cent of the total estrogen. These values are of the same order as those previously reported by Dingemanse and Tyslowitz (5) for the dog.

TABLE I

Excretion of Estrogens in Bile and Urine Following Injection of Estrone in Dogs

1 mg. (10,000 I. U.) of estrone was injected in each experiment.

All results are expressed in terms of the international unit of estrone. In each case marked % the value designates the per cent of the amount injected.

Experiment No.		1	2	3	4	5	6	7	8	9
Dog No.		1	1	2	2	3	4	5	5	5
Bile	Total	505	133	647	500	536	750	600	804	776
	Non-ketonic			623	500	536	805	556		
	Soluble in 0.3 M Na_2CO_3					229	315	196		<66
	Insoluble in 0.3 M Na_2CO_3					286	445	360		667
	Ketonic*			24	0	0	0	44		
	Total, %	5.1	1.3	6.5	5.0	5.4	7.5	6.0	8.0	7.8
Urine	Total	643	1348	804	833	750	970	760	652	865
	Non-ketonic					586	785	470		
	Ketonic*					164	185	290		
	Total, %	6.4	13.5	8.0	8.3	7.5	9.7	7.6	6.5	8.7
Bile and urine	Total, %	11.5	14.8	14.5	13.3	12.9	17.2	13.6	14.5	16.5

* Only the non-ketonic values were obtained by assay. Values for ketones were obtained by difference.

When the non-ketonic estrogens were fractionated by the method of Mather (23), from 9.0 to 42.7 per cent of the total non-ketones was found to be soluble in 0.3 M sodium carbonate, and from 53.2 to 91.0 per cent was insoluble in 0.3 M sodium carbonate. Only a small amount of substance remained in the fraction undergoing examination when this step was carried out. This made it impossible to inject an adequate number of animals in the assay procedure

and, therefore, we present these figures as approximations and not as absolute values.

Total Excretion—The total combined excretion in the urine and bile ranged from 11.5 to 17.2 per cent of the estrone injected. The values are of the same order as those reported by other workers. It is not possible to make strict comparisons between these results and those of other workers because of the differences in amount and type of material injected, and because of the differences in the animals used as experimental subjects. Furthermore, these experiments measure biliary excretion which under normal circumstances would be subject to reabsorption from the intestine. When Dingemanse and Tyslowitz (5) gave doses of estrone up to 20 times as great as those used in our experiments, they found *urinary* excretion in dogs to range from 7.2 to 16.5 per cent of the injected estrone.

DISCUSSION

It is readily evident that biliary excretion as measured in these experiments does not give a true picture of the final fate of injected estrone in the intact animal. Under normal conditions at least a part of whatever active compound found its way into the bile would be subjected to reabsorption from the intestine and to further action by the liver. These experiments do give information, however, concerning the relative excretion by the liver and the kidneys, and they serve as a starting point for further investigation of the chemical nature of the end-products of estrogen metabolism found in the bile.

It is significant that all, or practically all, of the active substance in the bile was non-ketonic. The fact that this was not true of the urinary estrogens indicates that some of the injected estrone reached the kidneys without contact with any of the tissues which participate in its metabolism. On the other hand, it is not impossible that the estrone which appeared in the urine may have been converted to a non-ketone and then reconverted to a ketone before its excretion (Heller (13); Fish and Dorfman (16)). In reaching the bile, however, the estrone was subjected to the action of the liver, which has been found to be particularly active in estrogen metabolism.

The appearance in the bile of a non-ketonic estrogen insoluble in

0.3 M sodium carbonate gives evidence from experimentation *in vivo* which tends to support the conclusion of Heller (13) that the liver converts estrone to α-estradiol. In his experiments it was found that the α-estradiol so formed was rapidly inactivated by the liver, but in the present studies it is evident that some of the substance which conforms to the solubility properties of α-estradiol escaped into the bile before inactivation had occurred.

Heller found, furthermore, that estriol was only slightly affected by the liver. Our work casts no light upon the point of its formation or upon its fate.

SUMMARY

The excretion of estrogens in the bile and urine, respectively, has been determined after the injection of estrone into female dogs. The biliary excretion ranged from 1.3 to 8.0 per cent of the injected estrone, and the urinary excretion was between 6.4 and 13.5 per cent. The minimum total excretion was 11.5 per cent and the maximum was 17.2 per cent.

In five determinations on bile extracts, the non-ketonic fraction constituted practically 100 per cent of the total estrogen present, whereas, in three determinations on urine extracts, only from 61.8 to 80.9 per cent of the total was non-ketonic. The biliary non-ketones were found to include an active substance insoluble in 0.3 M sodium carbonate as well as one soluble in this reagent.

The authors wish to express their appreciation of the cooperation of the following: Dr. Robert W. Virtue, who demonstrated to us the technique of preparation of the bile fistulas; Dr. Erwin Schwenk of the Schering Corporation, who furnished the estrone; and Dr. Alan Mather, who furnished detailed directions for his fractionation technique prior to its publication.

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MICRODETERMINATION OF CALCIUM BY PRECIPITATION AS PICROLONATE AND ESTIMATION OF THE PRECIPITATED CARBON BY MANOMETRIC COMBUSTION*

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The rapid and accurate gasometric procedure for microdetermination of organic carbon developed by Van Slyke, Page, and Kirk (13) and recently improved by Van Slyke and Folch (11) opens the way to an indefinite number of quantitative analyses in which substances are precipitated in centrifuge-combustion tubes with organic precipitants, and the amounts of the precipitates are measured by determinations of their carbon. This procedure is especially adaptable to microanalyses, because the combustion is accurate for samples containing amounts of carbon down to 0.1 mg., and precipitants can be used in which the organic component contains many carbon atoms for 1 atom of the element determined. Kirk (8) and Hoagland (5) have used this principle for micro phosphorus determinations in which strychnine phosphomolybdate, with 28.4 (empirical factor) times as much carbon as phosphorus, is precipitated and burned, permitting analyses of samples with less than 0.01 mg. of phosphorus. Similarly Hoagland has used combustion of benzidine sulfate for microdeterminations of sulfate (5) and of magnesium hydroxyquinolate for magnesium (6).

In the present method calcium is precipitated from aqueous solution as the picrolonate, $Ca(C_{10}H_7O_5N_4)_2 \cdot 8H_2O$, and the amount

* This paper was submitted by Frank J. Kreysa in partial fulfilment of the requirements for the degree of Master of Science in the department of Professor Joseph B. Niederl, Graduate School of Arts and Science, New York University.

of the precipitate is estimated from the CO_2 yielded by the combustion method of Van Slyke and Folch (11). The picrolonate is precipitated and burned without transfer in a Pyrex centrifuge tube. The fact that the picrolonate contains 20 carbon atoms to 1 calcium makes the method applicable to samples with amounts of calcium down to 0.02 mg.

Several authors have used picrolonate for quantitative determination of calcium. Dworzak and Reich-Rohrwig (4) weighed it as a precipitate. Alten, Weiland, and Knippenberg (1) developed a colorimetric method to measure the red color formed when the picrolonate was treated with bromine water. Bolliger (3) used known amounts of lithium picrolonate to precipitate the calcium, and estimated the picrolonate left in solution to calculate the amount precipitated by difference.

The properties of calcium picrolonate have been studied by Robinson and Scott (10) and by Dworzak and Reich-Rohrwig (4). There is some uncertainty as to whether there are 7 or 8 molecules of water of crystallization, but the water content is of no significance when the precipitate is measured by carbon analysis. The solubility in water at room temperature is reported by Robinson and Scott (10) to correspond to 5 mg. of calcium per liter. However, our results indicate that the presence of free picrolonic acid in solution depresses the solubility of the calcium picrolonate to a small fraction of this value.

To avoid resolution of any of the precipitate by wash water, washing is replaced by a complete drainage of the supernatant fluid. The picrolonic acid in the precipitated solution is so dilute (0.005 N), and the amount of it which remains adherent to the minute precipitate and to the clean walls of the tube is so slight and constant, that the carbon in it can be included without significant error in the correction for reagents obtained by blank analyses.

Apparatus

1. For the gasometric determination of carbon in the precipitate the Van Slyke-Neill (12) manometric apparatus is used, with the accessories for combustion described by Van Slyke and Folch (11). The shape of the combustion tube, however, is altered by tapering its bottom into a cone as shown in Fig. 1, so that it can serve also

as a centrifuge tube. The bottom of the tube must be of the dimensions and shape indicated in Fig. 1, in order to hold the precipitates and to permit complete decantation of the supernatant fluid. The form of combustion-centrifuge tube shown in Fig. 1 is somewhat more convenient than that used by Hoagland (5). Complete decantation is easier, and the combustion can be carried out with a free flame, as in Van Slyke and Folch's description of the combustion (11), without need of the protecting device between the flame and tube which Hoagland found necessary.

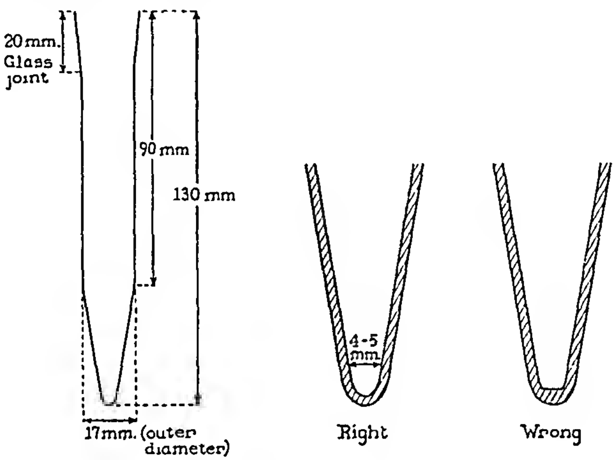

FIG. 1. Combustion-centrifuge tube

2. A drying oven with a temperature of 100–105°.

3. For use when the calcium-containing material contains organic matter and must be ashed, an electric muffle in which the temperature can be set at 500°.

4. Pyrex glass stirring rods of about 2 mm. diameter to mix the precipitating solutions in the combustion-centrifuge tubes. It is well to make a small button on one end of each rod, and always use the other end for the stirring, in order to avoid contaminating the solutions with organic matter from the fingers.

The combustion-centrifuge tubes and the rods are cleaned with hot chromic acid mixture as described by Van Slyke and Folch ((11) p. 533). However, the desiccators with calcium chloride used by Van Slyke and Folch cannot be used when the tubes

are to be used for calcium determinations. The calcium chloride can be replaced by phosphorus pentoxide, or the washed tubes can simply be inverted in a covered beaker or wide mouthed Erlenmeyer flask and dried in an oven.

Reagents

1. *Approximately 0.008 N picrolonic acid.* The purity of the picrolonic acid used is important, in order to obtain properly crystalline calcium picrolonate precipitates. Some impure preparations of picrolonic acid have been tried which regularly gave amorphous precipitates of calcium picrolonate, impossible to handle in the centrifuge tubes by the technique employed. A preparation of Kahlbaum's picrolonic acid "for analysis" could be used without purification. Other preparations had to be recrystallized.

To prepare the solution 2.1 gm. of picrolonic acid and 1 liter of water are placed in an Erlenmeyer flask covered with a watch-glass to retard evaporation, and are heated for 2 or 3 hours on a steam bath. The flask is rotated occasionally to assist solution. The solution is cooled to room temperature and filtered through a calcium-free filter paper to remove any undissolved residue. The solution is kept in an ice box, so that any material which is insoluble at 0° will precipitate before the solution is used.

2. *Approximately 0.04 N hydrochloric acid.*
3. *Concentrated sulfuric acid in a dropping bottle.*
4. *Reagents for the Van Slyke-Folch combustion* (11).

Procedure

A sample of solution containing 20 to 120 γ of calcium is measured into the combustion-centrifuge tube and concentrated to dryness by placing the tube in the oven at 100–105°. If any organic matter is present, it is then destroyed by adding a drop of concentrated sulfuric acid and ashing at 480–520° in the electric oven.

When serum is analyzed, 0.2 or 0.5 cc. is measured into the centrifuge-combustion tube and 1 drop of concentrated sulfuric acid is added. The tube is put into the oven at 100–105° for 2 hours, and is then transferred to the electric furnace, where the temperature is raised slowly to 480–520°. It is left overnight at this temperature, which accomplishes the ashing with-

out softening the Pyrex glass. If particles of carbon still remain in the ash, the tube is returned to the furnace until the ash is white, but the initial 12 to 14 hours usually suffice. The added sulfuric acid accelerates the destruction of organic matter and also serves to protect the glass against the etching which might result if an alkaline ash or one high in chlorides were formed.

To dissolve the ash 1 cc. of 0.04 N hydrochloric acid is added. One of the cleaned stirring rods is used to loosen up the ash, and is left in the tube while the latter is immersed in boiling water for 10 minutes. Then 1 cc. of hot distilled water is added and the heating is continued for another 10 minutes to complete solution of the ash.

The solution is cooled to room temperature and 2 cc. of the ice-cold filtered solution of picrolonic acid are added. The liquids are stirred occasionally with the rod until crystals of calcium picrolonate appear, which occurs in 3 to 5 minutes.

After a half hour at room temperature the tube is cooled to near 0° by immersion in ice water, and is kept at this temperature for an hour and a half. During the first half hour it is stirred two or three times. The stirring rod is then withdrawn and rinsed into the tube with 1 cc. of the 0.008 N picrolonic acid solution. The tube is capped and left for an hour longer at 0° to complete the crystallization.

The tube is then centrifuged for 15 minutes at 3000 R.P.M. and the supernatant fluid is removed by suction through a fine curved capillary. To avoid loss of any of the crystals floating on the surface film, suction is not started until the capillary is immersed well below the surface. Then as the fluid is withdrawn the particles in the surface film stick to the wall of the centrifuge tube and are not lost. Suction is stopped when 0.2 to 0.3 cc. of fluid remains over the precipitate.

To drain off the rest of the supernatant solution the tube is inverted to an angle of about 30° with the horizontal and the lip is rested on a wet towel or filter paper. If a drop of liquid adheres in the narrow bottom of the tube, it is touched with a curved platinum wire, which breaks the surface film and starts drainage. After 2 minutes at the 30° angle the tube is shifted to a nearly vertical position and left there for 15 minutes to complete the drainage. The mouth of the inverted tube is sprayed with a few

drops of water to remove adherent picrolonic acid solution from the lip. Drainage in this manner leaves in the tube a uniform film of approximately 0.025 cc. of liquid. This film contains 0.031 mg. of picrolonic acid, equivalent to 2.3 γ of calcium. Variations in the film are not great enough to cause variations of more than ± 1 mm. in the blank, equivalent to 0.2 γ of calcium.

After the tube has drained, the carbon in the precipitate is determined by combustion as described by Van Slyke and Folch (11), the centrifuge tube serving as combustion tube. The pressure readings are made with the gases at 2 cc. volume.

As a routine we have dried the tubes by immersion in a steam bath before the combustion, but this is not necessary, as the slight film of aqueous solution does not contain enough water to interfere with the efficiency of the combustion fluid.

Blank Analysis—3 cc. of the 0.008 M picrolonic acid and 2 cc. of water are placed in a centrifuge-combustion tube which has been cleaned by use in a previous combustion or by heating in chromic-sulfuric acid. The picrolonic acid solution is decanted and the tube is drained exactly as in the analyses, and is subjected to combustion. The value of $p_1 - p_2$, measured with gas volume at 2 cc., obtained in this blank combustion is the c correction. It includes a correction of about 10 mm. for the carbon in the picrolonic acid of the film left in the tube after draining.

Calculation

The micrograms of calcium in the sample are calculated by multiplying the CO_2 pressure, P_{CO_2}, from the burned precipitate by a factor given in Table I.[1]

$$\text{Micrograms Ca} = P_{CO_2} \times \textit{factor}$$

$$P_{CO_2} = (p_1 - p_2 - c)$$

p_1 and p_2 are the manometer readings before and after absorption of the carbon dioxide and c is the value of $p_1 - p_2$ obtained in the blank analysis.

[1] The factors for micrograms of calcium given in Table I are the factors for mg. of carbon of Van Slyke and Folch ((11) p. 529) multiplied by 1000 $\times$ 0.16686,

$$0.16686 = \frac{40.08}{240.20} = \frac{\text{weight of 1 gm. atom calcium}}{\text{weight of 20 gm. atoms carbon}}$$

TABLE I

Factors for Calcium Calculation

Temperature	Factor; micrograms Ca indicated by 1 mm. P_{CO_2}, when a = 2.000 cc.*
°C.	
10	0.2460
11	46
12	33
13	21
14	09
15	0.2398
16	86
17	76
18	64
19	53
20	41
21	31
22	19
23	09
24	0.2299
25	89
26	79
27	69
28	59
29	51
30	41
31	31
32	23
33	14
34	04
35	0.2196

* If a is not exactly 2 cc., multiply the factor in the table by $a/2$.

EXPERIMENTAL

I. Determination of Calcium in Standard Calcium Solutions with from 20 to 100 γ of Calcium

A stock solution containing approximately 1 mg. of Ca per cc. was prepared as follows: Selected crystals of Iceland spar were washed in dilute hydrochloric acid (about 0.1 N), then with water,

and were dried over phosphorus pentoxide. An accurately weighed sample of about 2.5 gm. of these crystals was transferred to a 1 liter volumetric flask, dissolved in 15 cc. of concentrated hydrochloric acid, and diluted with water to 1 liter.

From this stock solution, 1, 2, 3, 4, and 5 cc. were accurately pipetted into 100 cc. volumetric flasks and diluted to the mark with water to make the dilute standards containing 10 to 50 γ of Ca per cc.

For the analyses in Table II, 2 cc. portions of the standard solutions were pipetted into the centrifuge-combustion tubes followed by 2 cc. of ice-cold 0.008 N picrolonic acid solution, and the procedure of analysis was followed from that point as described above for routine analyses.

The results are summarized in Table II.

II. Serum Analyses: (1) Comparison of Picrolonic Acid Method with Results Obtained by Microtitration of Calcium Oxalate by Potassium Permanganate; (2) Recovery of Added Calcium

1. Calcium determinations on blood serum were performed as described in this paper and the results obtained are summarized in Table III.

For the microtitration with permanganate 2 cc. samples of serum were analyzed as described in Peters and Van Slyke (9), with washing of the oxalate by Clarke and Collip's decantation method.

2. Results from recovery of calcium added are summarized in Table IV.

Estimation of Calcium in Low Calcium Tap Water by Combustion of Calcium Picrolonate

According to Dworzak and Reich-Rohrwig (4) calcium can be determined gravimetrically as picrolonate in ordinary drinking and tap water. The silicic acid and iron to be expected do not interfere. These authors determined calcium in Vienna's mineral waters, treating them directly with picrolonic acid without any preliminary operations.

For control determinations by titration, 100 cc. portions of the water were concentrated to dryness on the steam bath and the residue was redissolved in 1 cc. of 1 N hydrochloric acid. The

Determination of Calcium in Standard Solutions

Calcium content of sample	P_{CO_2}	a*	Temperature	Factor from Table I	Calcium	
					Found	Error
γ	mm.	cc.	°C.		γ	per cent
100.8	444.7	2.006	28.6	0.2261	100.5	−0.3
100.8	442.6	2.006	28.8	59	100.0	−0.8
80.1	360.6	2.000	32.0	23	80.2	+0.1
80.1	359.0	2.000	32.3	20	79.8	−0.4
60.1	268.3	2.000	29.6	45	60.2	+0.2
60.1	267.6	2.000	29.5	46	60.1	±0.0
40.1	179.7	2.000	29.2	49	40.4	+0.8
40.1	177.6	2.000	28.9	52	40.0	−0.3
20.0	88.5	2.000	31.8	24	19.7	−1.5
20.0	90.9	2.000	32.0	23	20.2	+1.0

* Exact volume at which CO_2 pressures were measured.

Table III

Comparison of Calcium Determination in 2 Cc. Samples of Serum by Oxalate Titration Method with Determinations in Samples of 0.5 and 0.2 Cc. by Present Method

Strain No.	Calcium content by present method						Calcium by titration, per 100 cc. serum
	Volume of sample	P_{CO_2} (duplicates)	a	Temperature	Ca in sample	Ca in 100 cc. serum (average of duplicates)	
	cc.	mm.	cc.	°C.	γ	mg.	mg.
1*	0.5	123.9	2.000	26.0	28.3		
	0.5	126.6	2.000	26.2	28.8	5.72	5.61
2	0.5	220.6	2.000	29.0	49.7		
	0.5	218.2	2.000	29.0	49.1	9.88	9.94
3	0.5	226.8	2.006	31.3	50.7		
	0.5	225.8	2.006	31.4	50.5	10.12	10.16
4	0.5	235.0	2.006	27.4	53.4		
	0.5	232.0	2.006	27.5	52.7	10.62	10.58
5	0.5	242.6	2.006	30.4	54.5		
	0.5	243.8	2.006	30.5	54.7	10.92	11.04
6*	0.2	60.2	2.006	31.4	13.5		
	0.2	61.8	2.006	31.4	13.8	6.85	6.75
7	0.2	100.0	2.006	30.6	22.4		
	0.2	97.9	2.006	30.9	21.9	11.1	11.04
8	0.2	88.7	2.006	30.9	19.8		
	0.2	90.4	2.006	31.1	20.2	10.0	10.16

* Nephritic with low serum calcium.

solution was washed into a centrifuge tube and brought to pH approximately 5 by adding sodium acetate. The calcium was precipitated as oxalate which was washed three times by centrifugation with saturated calcium oxalate solution, and titrated as described by Peters and Van Slyke (9). Triplicate determinations indicated 12.42, 12.41, and 12.51, average 12.45, mg. of Ca per liter of water.

TABLE IV

Recovery of Calcium Added to Serum

Serum No.	Calcium present in 0.5 cc. serum before addition	Calcium added per 0.5 cc. serum	Calcium found after addition, per 0.5 cc. serum	Recovery of added Ca	
				Per 0.5 cc. serum, average of duplicates	Per cent of added Ca
	γ	γ	γ	γ	
1	50.6	50.4	101.5		
			101.9	51.1	101.3
2	52.5	50.4	104.2		
			104.5	51.7	102.5
3*	28.5	40.0	68.1		
			67.9	39.5	98.8
4	54.6	50.4	103.9		
			103.8	49.3	97.8

* Nephritic with abnormally low serum Ca.

TABLE V

Determination of Calcium in New York City Tap Water

Calcium determined in 100 cc. samples by permanganate titration = 12.45 mg. per liter.

Volume of sample	P_{CO_2}	a	Temperature	Factor	Ca in sample	Ca per liter
cc.	*mm.*	*cc.*	*°C.*		γ	*mg.*
2.0	134.5	2.006	27.8	0.2268	25.0	12.52
2.0	134.5	2.006	27.9	67	25.0	12.52

For microanalyses by picrolonate combustion 2 cc. portions of New York City tap water were treated in the same way as the calcium standard solutions. The results given in Table V serve as an example of a water of relatively low calcium content.

Remarks

The 0.008 M picrolonic acid solution used as precipitant is approximately saturated at 0°. For the exact solubility of pure picrolonic acid in water Hugouneng, Florence, and Couture (7) gave 1.2 gm. per liter at 17°, but other authors (1, 4) have used 0.01 M solutions (2.64 gm. per liter) as analytical reagents.

If the concentration of free HCl were too great in the precipitating solution, free picrolonic acid would be precipitated with the calcium picrolonate. We have found that HCl up to 0.02 N concentration does not precipitate picrolonic acid under the conditions of the present analysis, but that 0.05 N HCl would cause error from precipitation of free picrolonic acid. The conditions for redissolving the ash of serum are designed to keep the HCl concentration within 0.02 N at the time of precipitation.

Under the conditions used for serum analysis there is no interference from the amounts of magnesium present.

The chief difficulty encountered in making the method practical was in ascertaining the conditions to insure a dense crystalline precipitate, suitable for centrifuging and decanting. Pure picrolonic acid was found to be essential; one commercial preparation gave only amorphous precipitates until it was purified. Furthermore, the concentrations of the calcium and the picrolonic acid and the temperature of the solution influence the character of the precipitate. The prescribed stirring with a glass rod is necessary to obtain uniformly the crystalline form of the precipitate.

In ashing serum, temperatures up to 520° could be used without softening the Pyrex glass, and repeated carbon determinations on the acid ash gave negative results. Temperatures much higher than 520° soften the glass enough to spoil the fit of the ground glass joint of the tube. Baernstein and Grands (2), in a paper which has appeared since this work was completed, use a similar ashing technique, but with silica tubes and a temperature of 600°, which gave complete ashing in 3 hours. If many calcium determinations were to be run routinely, the time saved by substituting silica for Pyrex would probably be worth the extra cost.

SUMMARY

A micromethod for calcium is described in which the calcium is precipitated as picrolonate and the precipitate, containing 20 atoms of carbon to 1 of calcium, is estimated from the carbon, which is determined by the rapid manometric wet combustion method of Van Slyke and Folch. Precipitation and combustion are done without transfer in a single centrifuge-combustion tube.

The method serves for estimation of smaller amounts of calcium than can be determined accurately by the usual microprocedures based on titration of the oxalate; 0.2 mg. of calcium or 0.2 cc. of serum suffices for an analysis.

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STUDIES ON THE HEMOLYTIC STREPTOCOCCUS

IV. FURTHER PURIFICATION AND CONCENTRATION OF SCARLET FEVER TOXIN *

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In a previous paper (1), the preparation of a partially purified erythrogenic toxin of scarlet fever was described. This fraction contained 10,000,000 skin test doses per mg., but had chemical properties similar to non-erythrogenic fractions from non-scarlatinal strains. Further studies have indicated that a heat-coagulable protein, present only in the toxic fractions, is actually the specific scarlet fever toxin. The active protein contained about 200,000,000 skin test doses per mg. The toxin was about 50 per cent pure in the best preparations.

In the present paper the methods of preparation and identification of the heat-coagulable protein will be presented. The separation of pure toxin by means of the Tiselius electrophoresis apparatus and the chemical and physical properties of the protein will be described in another paper (2). The essential principles for the preparation of the toxin were (*a*) the use of a diffusate medium which had passed by dialysis through No. 600 cellophane for the culturing of the streptococcus, and (*b*) the separation from these cultures of a filtrate containing a non-dialyzable toxin. Two factors have been largely responsible for increased bacterial growth and enhanced toxin production; namely, first, the replacing of the meat infusion broth by a tryptic digest broth modeled after the methods of O'Meara (3) and Pope and Linggood (4). A modification in the method of adding the trypsin was made by the substitution of whole pancreas for Cole and Onslow's acid alcohol extract

* This work was supervised by Dr. Maud L. Menten.

of pancreas. The second factor was the use of culture neutralization similar to those methods practiced by Mueller and Klise (5), Hooker, Follensby, Claxton, and Tayian (6), and others.

Preparation of Medium.—The basic broth for dialysis was prepared in the following manner. For 80 liters of broth, 60 pounds of ground beef heart, 30 pounds of ground beef pancreas (fresh or frozen), and 40 liters of water (tap or distilled) were used. 20 liters of water were added to the beef heart and heated to 80° in a steam cauldron in order to destroy antitrypsin. After addition of another 20 liters of water and cooling to 50° the mixture was adjusted to pH 8 with Na_2CO_3, phenol red being used on a spot plate. 6 pounds of pancreas were added initially and at the end of the 1st, 2nd, 3rd, and 4th hours. After each addition, the reaction was adjusted to pH 8. The initial adjustment required approximately 240 gm. of Na_2CO_3 and each subsequent adjustment required about 60 gm. Digestion was allowed to proceed between 42–50° for 5 hours, at which time the digested mixture was made acid to phenol red with approximately 750 ml. of concentrated HCl and placed in a steam cauldron. The mixture was boiled for 15 minutes, cautiously because of foaming. The steam was turned off and the whole was allowed to settle for 5 minutes. The spigot at the bottom of the cauldron was opened and the broth drawn off through the packed layer of meat, filtered through glass wool, and then adjusted to pH 7.8 with 10 per cent NaOH (approximately 1.5 liters being required). The fluid was then distributed between two containers of 35 liters capacity each and left in the refrigerator overnight to allow the phosphate to settle out. The following morning the supernatant broth was siphoned off and distributed into twelve cellophane bags, each of which was placed in 5 liters of water for dialysis as described in detail elsewhere (1). After 24 hours dialysis in the ice box, the clear golden yellow diffusate, which had passed through the cellophane, was removed and pooled. A second dialysis with each bag was made against 2.5 liters of water and these diffusates added to the first portion. After completion of dialysis, 0.05 per cent of dextrose was added to the total diffusate and the broth was autoclaved at 10 pounds (115°) for 30 minutes. The dialyzed medium contained only 35 gm. of solids per liter as compared to 110 gm. per liter for the whole digest broth. For the preparation of one lot of toxin, instead of the

described tryptic diffusate, a dried tryptic medium prepared by Difco, Detroit, was used. The toxin derived from this medium contained considerable pigment.

Inoculum of the NY5 strain of streptococcus was prepared and transferred into the diffusate medium as previously described (1). 10 to 80 liters of diffusate medium were cultured at one time. The cultures were incubated overnight at 30–37.5°. Wide range of temperature made little difference in bacterial growth or toxin production. In the morning 100 ml. of a 60 per cent glucose solution containing 40 mg. of phenol red were added to each flask containing 4.5 liters of medium. The lactic acid developing during bacterial growth was neutralized to between pH 7 and 7.4 periodically with NaOH or by immediate addition of $NaHCO_3$. A total of 50 ml. of 10 per cent NaOH per liter of culture was the amount usually needed for periodic neutralization during the 5 hour period of rapid growth. If the $NaHCO_3$ was used, 600 ml. of a saturated (10 per cent) solution, sterilized by filtration through a 10 $\times$ 2 inch Berkefeld N filter, were added to each flask (4.5 liters) of overnight culture. This amount is sufficient for adequate neutralization of the lactic acid formed during bacterial growth. Our experiments have shown that as high as 3 per cent $NaHCO_3$ may be used in tryptic diffusate broth without inhibition of bacterial growth. The use of 4 per cent $NaHCO_3$ proved to be inhibitory. 24 hours after the beginning of neutralization the cultures usually became quite acid, about pH 5.8. With the diffusate medium, the growth of bacteria obtained was always exceedingly profuse. A modified Gates-Feemster (7) nephelometer was used for measuring bacterial growth. Without neutralization of the culture, readings of 70 to 90 were obtained, but with neutralization of the acid developing with bacterial growth readings as high as 270 to 400 were obtained. Similarly, the amounts of toxin increased in almost direct proportion to the growth. Filtrates of neutralized cultures contained 200,000 skin test doses per ml. A sterile filtrate was obtained from the cultures by passage through a 10 $\times$ 2 inch Berkefeld V filter equipped with an automatic siphon arrangement.

Separation of Toxin—The method for the separation of the toxin from the filtrate was the same as that previously published (1), and consisted of adsorption at pH 4.0 with Lloyd's reagent, elution

with alkaline buffer, precipitation of the eluate with ammonium sulfate, and dialysis of the precipitate in a cellophane bag. Several improvements have facilitated the procedure: Addition of 2 gm. of Lloyd's reagent per liter of filtrate was required to insure complete adsorption, and 65 gm. of solid ammonium sulfate per 100 ml. of eluate were used instead of a saturated solution for precipitation.

In many of the earlier preparations, clupein sulfate (British Drug Houses) was added to the final dialyzed solution of toxin and the resulting precipitate discarded. This reagent removed a nucleoprotein-like substance precipitable by acid (2) and also cleared the solution of much brown pigment. The nucleoprotein-like substance was found in cultures neutralized either by NaOH or $NaHCO_3$. Its significance is not known. The use of clupein sulfate was omitted in later preparations, because it added a possible interfering protein constituent.

Preliminary Testing of Isolated Toxic Fraction—The dialyzed solution containing the toxin could be separated into two parts by heating. The heat-coagulable part was shown to contain the erythrogenic toxin. In the earlier preparations the protein coagulum was obtained by heating without addition of salt. Later, with more highly purified preparations, it was necessary to add ammonium sulfate in order to obtain a heat-coagulable precipitate.

For identification and determination of the amount of heat-coagulable toxic protein, the following procedure was used. To 0.2 ml. of the test solution, 2 drops of saturated ammonium sulfate were added, and the mixture heated 15 minutes in an oven at 110°. A coagulum indicated toxin was present. When no salt had been added, the coagulum and the non-heat-coagulable residue were dried and weighed on the Kuhlmann micro balance. Equivalent amounts of coagulum were centrifuged and resuspended in water, and used as a means of establishing a standard turbidimetric reading on a Klett photoelectric colorimeter. Subsequently, approximate determinations of weights of toxic protein were made by the turbidimetric method in order to save material and avoid difficulties involved in microprocedures. From the weight of heat-coagulable protein, or the turbidity readings, approximate preliminary dilutions of toxic protein could be made for skin testing. Skin tests compared with controls obtained by commercial Dick

toxin have indicated that the heat-coagulable protein contained about 200,000,000 skin test doses per mg.

Proof That Heat-Coagulable Protein Contained Scarlet Fever Toxin—As indicated elsewhere (2) the heat-coagulable protein was precipitable by trichloroacetic acid, whereas the non-heat-coagulable residue was not precipitated. Heat coagulation denatured the toxin irreversibly. Trichloroacetic acid denatured the toxin, but not completely. The protein precipitated with 5 per cent trichloroacetic acid was insoluble in water. The precipitate was triturated with ether, washed with water, and cautiously dissolved by the addition of dilute NaOH to neutrality. The trichloroacetic acid was dialyzed out from the non-acid-precipitable solution after removal of the precipitate. The dissolved precipitate and the dialyzed residue were skin-tested. The toxin, considerably diminished in titer, was present only in the dissolved precipitate.

Absence of Hemolysin and Fibrinolysin Production in Diffusate Medium—Hemolysin and fibrinolysin production of the NY5 strain in the diffusate and the whole tryptic digest broth was tested. There was little or no hemolysin production in the diffusate. This strain also failed to produce fibrinolysin in the diffusate medium, which is not surprising, since the NY5 strain is poorly fibrinolytic. Therefore, fractions containing hemolysin or fibrinolysin were not anticipated in the final purified toxic preparations. However, it is noteworthy that hemolysin production by the NY5 strain in undialyzed tryptic digest broth gave a high lytic titer, *viz.* 1 + hemolysis with a lysin dilution of 1:2048, and 0.5 ml. of 5 per cent red blood cells (human). This hemolysin was free in the supernatant fluid of a centrifuged culture.

Results

A total of thirty-five preparations of scarlet fever toxin was made by the outlined methods. In all instances a heat-coagulable toxic protein was obtained in the final purified fraction. The amounts of specific protein varied from 0.2 to 1.0 mg. per liter of filtrate. There was a close correlation between the weight of protein and the number of skin test doses; namely, about 200,000,000 skin test doses per mg. The percentage of heat-coagulable protein in the final fractions varied. Preparations treated with

clupein were about 50 per cent pure, but the percentage of protein in untreated fractions was less. The final products were highly colored with brownish pigments unless treated with clupein.

Numerous attempts to separate chemically the protein from pigment or non-heat-coagulable material were not very successful. Chemicals, such as trichloroacetic acid, separated the protein but there was denaturation. Fractional precipitation with ammonium sulfate, or by the use of alcohol, acetone, or dioxane did not separate appreciable amounts of non-heat-coagulable material. Attempts made to "crystallize" the protein were abandoned, owing to the small amounts of substance available.

Results with Other Strains of Hemolytic Streptococci—Toxin separated from filtrate prepared from another strain of streptococcus of scarlet fever, Dick 2, possessed properties identical with those characteristic of the NY5 strain. Heat-coagulable protein was separated and skin tests indicated its activity to be comparable to that of the protein obtained from the NY5 strain. On the other hand, two small lots of filtrate from two non-erythrogenic non-scarlet fever strains gave no yield of a heat-coagulable toxic protein.

DISCUSSION

2 years of extensive study of thirty-five preparations of heat-coagulable toxic protein have indicated its identity with the true scarlet fever (erythrogenic) toxin. A diffusate medium which has been dialyzed through cellophane assures the bacterial origin of the non-dialyzable, heat-coagulable protein. Toxic activity roughly paralleled the quantities of protein present in the heat-coagulable fraction. No heat-coagulable protein was isolated from non-scarlatinal, non-erythrogenic strains of hemolytic streptococci. No hemolysin or fibrinolysin production occurred in the diffusate mediums and these toxins at least did not complicate the isolation of scarlet fever (erythrogenic) toxin. Separation of the protein by trichloroacetic acid precipitation and resolution indicated either that the toxin was associated with this protein or that the protein was the toxin itself. Evidence for the latter assumption is adduced in the following paper (2).

The scarlet fever toxic fractions obtained by Barron, Dick, and Lyman (8) and by Koerber and Bunney (9) have low potency as compared to our heat-coagulable protein. The toxin studied by

Barron, Dick, and Lyman contained 20,000 to 30,000 skin test doses per mg. compared to 200,000,000 skin test doses per mg. for the heat-coagulable protein. The "protein-free" scarlatinal toxin prepared by Koerber and Bunney contained 100,000 skin test doses per 0.01 mg. of nitrogen compared to 15,000,000 skin test doses per 0.01 mg. of nitrogen for the heat-coagulable protein.

SUMMARY

A heat-coagulable protein apparently identical with scarlet fever (erythrogenic) toxin was separated from filtrates of cultures in improved diffusate mediums. The toxic protein contained about 200,000,000 skin test doses per mg. and was approximately 50 per cent pure. It was not obtained in crystalline form.

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STUDIES ON THE HEMOLYTIC STREPTOCOCCUS

V. THE ELECTROPHORETIC ISOLATION OF THE ERYTHROGENIC TOXIN OF SCARLET FEVER AND THE DETERMINATION OF ITS CHEMICAL AND PHYSICAL PROPERTIES*

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A chemical procedure has been developed which yields concentrated scarlet fever toxin of a high degree of activity (1). Preparations obtained by this method contained a heat-coagulable protein which was apparently identical with scarlet fever erythrogenic toxin; this protein was at best only 50 per cent pure. The toxin preparations (hereafter referred to as chemically isolated toxin, and designated Toxin CI) have now been subjected to electrophoretic fractionation. A heat-coagulable protein was separated which alone carried the toxic activity, and which was homogeneous with respect to both electrophoresis and sedimentation in the ultracentrifuge. The chemical and physical properties of this heat-coagulable protein were determined, and data were also obtained on the other constituents accompanying the toxic protein.

Preparation of Materials—The methods used for obtaining the concentrated solutions of Toxin CI were those already described (1). The cultures were kept neutral with NaOH. The use of clupein for purification was omitted to avoid the interference of an additional protein constituent. The sources of the four lots of toxin, P, A, B, and C, submitted for electrophoresis were as follows: Lot P was prepared from filtrates of cultures grown in the diffusate

* This work has been carried out in cooperation with Dr. Maud L. Menten of the Children's Hospital and the University of Pittsburgh, Pittsburgh.

of dried tryptic digest broth specially made for us by Difco (1). The amount of brownish pigment was excessive. The remaining lots were recovered from growths in diffusates of undried tryptic digest broth. Lot A was pooled material from several preparations. Lots B and C were prepared from single large quantities of diffusate, and the final toxin solutions were concentrated to small volumes by evaporation from the frozen state.[1] All samples were shipped between Pittsburgh and Philadelphia or Newark, Delaware, by air mail.

Electrophoresis of Chemically Isolated Toxin Preparations; Composition and Mobilities—Electrophoresis of Toxin CI was performed in standard Tiselius equipment (2). The movement of

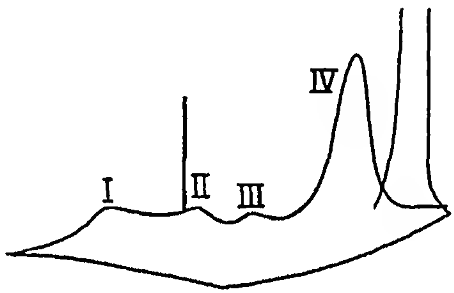

FIG. 1. Electrophoretic diagram of chemically isolated scarlet fever toxin. Lot A; potential gradient, 4 volts per cm.; times of migration, 0 and 2 hours. Descending boundaries, cathode arm. The vertical line shows the position of the pigment boundary (colored solution to the left, colorless solution to the right). The roman numerals represent constituents.

the boundaries was recorded by the schlieren scanning method (3). Before electrophoresis, each lot was either concentrated by evaporation from the frozen state or diluted by the addition of buffer solution to the volume necessary to fill the electrophoresis cell, and then dialyzed to equilibrium against large volumes of a buffer solution of pH 7 containing 0.15 M NaCl and 0.02 M phosphate. A typical schlieren diagram after migration for 2 hours at a potential gradient of 4 volts per cm. is reproduced in Fig. 1.

Three of the four toxin preparations were resolved into five constituents: I, II, III, IV, and a brownish pigment, unnumbered,

[1] The evaporation was carried out by Dr. Donald Wilson of the Physiological Chemistry Department, School of Medicine, University of Pittsburgh.

which was adjacent to Constituent II but was not associated with it; all migrated toward the anode in this particular buffer. The concentration of the pigment was too low to influence the refractive index diagrams, and the migration was strongly inhomogeneous. Lot C was similar to the other lots except that it contained a sixth constituent (N); after the first fractionation this precipitated out, presumably because it was insoluble in the absence of one or more of the faster migrating constituents, and it was observed thereafter only as a turbidity boundary. The turbidity was removed by centrifuging in the multispeed head of the International centrifuge for 10 minutes at 9000 R.P.M. with dry ice placed in the casing to maintain a low temperature.

The mobilities of the colorless constituents, calculated from the migration of the descending boundaries in the cathode arm of the U-tube (4), are listed in Table I (1–3, 5). The relative concentrations of these constituents and the absolute concentration of refractive material in each lot are given in Table II; these were calculated from the areas of the schlieren scanning diagrams upon the assumption that the specific refractive increment of each constituent is 0.0018, and were corrected for the dilution or concentration which preceded electrophoresis.

The turbidimetric analyses (1) for the heat-coagulable protein in each lot are included in Table II. As will be shown later, both Constituent II and Constituent IV are coagulated by heat. Consequently, the refractometric estimates of total concentration must be multiplied by the sum of the percentages of Constituents II and IV for comparison with the turbidimetric values. Our experience has indicated that the turbidimetric method of analysis does not give an exact measure of the weight of heat-coagulable protein. The inaccuracy of the method is probably due to several factors, chief of which are the adsorption of pigment by the coagulum and the insensitivity of the turbidimeter (a Klett photoelectric colorimeter) to the small amounts of coagulum available. Variations in the size of the coagulated particles may, likewise, cause appreciable differences in the turbidimetric readings. As can be seen, the values obtained turbidimetrically were high compared with those calculated from the refractive index in the electrophoretic determination. The latter figures, although based on an assumed value for the refractive index, were probably not more than 25 per

cent in error. However, the values obtained by turbidimetric analyses for different unfractionated samples were suitable for comparison, and were sufficiently within the range of the true

TABLE I

Mobilities of Constituents of Chemically Isolated Scarlet Fever Toxin (Toxin CI)

Buffer, 0.15 M NaCl, 0.02 M phosphate, pH 7. Net charge negative in all cases.

Run No.	Content of solution	Constituent No.				
		I	II	III	N	IV (toxin)
1	Lot P	10*	8*			2*
2	" A	10.3	6.8	5.3		1.9
3	" B	10.0	6.8	5.2		2.2
4	A and B	10.6	6.7	5.0		2.1
5	" " "		7.0	5.1		2.3
6†	Lot C	9.8‡	5.2	3.4	2.5	0.9
7	Predominantly C, small amounts A and B	9.9	6.5	5.3	2.9§	1.9
8	" "					1.9
9	" "					1.9
10	" "				3.2§	2.0
11	C, A, and B					1.93‖

* Value only approximate.

† In this run some disturbance of unknown origin (perhaps a slow leak through one of the rubber joints of the apparatus) caused a fairly uniform movement of the solution as a whole through the cell in the direction opposite to that of migration, and diminished the apparent movement of the boundaries through the cell. The extent of separation of the boundaries was normal. Approximately normal values for the mobilities are obtained by adding a constant (1.0 to 1.5) to each of the figures.

‡ From measurements of the migration of the ascending boundaries in the positive arm of the U-tube.

§ From measurements of the movement of the turbidity boundary.

‖ Calculated from the positions of the ordinates bisecting the curve areas; the most reliable figure.

values for calculation of the appropriate preliminary dilutions for clinical skin tests.

The similarity of the different lots of material isolated from the bacterial filtrates was clearly shown by the electrophoretic pat-

terns, particularly of the more concentrated Lots A, B, and C. However, considerable variation was observed in the relative quantities of the four constituents from lot to lot (Table II); this was probably the result of differences in the growth of the bacteria or of slight variations in the details of the preparation.

Electrophoretic Fractionation; Identification of Constituents—For the purpose of electrophoretic fractionation, migration was allowed to proceed, with compensation as required, until the boundaries for Constituents I and IV in each arm of the U-tube were separated by the entire cell height. The fractions separated by the electrophoretic fractionation of Lots P and B were returned to the

TABLE II

Composition of Four Lots of Chemically Isolated Scarlet Fever Toxin (Toxin CI)

Toxin lot	Percentage composition*					Total concentration*	Heat-coagulable substance†
	Constituent I	Constituent II	Constituent III	Constituent N	Constituent IV (toxin)	*mg. per ml.*	*mg. per ml.*
P	13	25			62	2.6	0.7
A	13	17	13		57	9.5	11.0
B	18	37	21		24	5.4	5.0
C	24	13	9	7	47	8.3	9.5

* Determined by integration of the schlieren scanning diagrams, assuming that the specific refractive increment of each constituent is 0.0014.

† Determined by turbidimetric analysis.

Children's Hospital, Pittsburgh, and analyzed for heat-coagulable protein by the turbidimetric procedure; from the turbidimetric values, dilutions were calculated for Dick tests on human beings. The turbidimetric analyses and the skin test data for the solutions collected from the compartments of the electrophoresis cell, for both Lot P and Lot B, are listed in Table III together with the refractometric estimates of the concentrations of each of the four constituents. The skin test data for Lot B show that the toxic activity is associated with Constituent IV alone. The lower anode fraction of Lot P showed somewhat higher activity, and the upper cathode fraction lower activity, than would be expected from the estimated content of Constituent IV, but owing to the low concen-

TABLE III

Turbidimetric and Skin Test Data on Fractions Separated by Electrophoresis

Fraction	Upper anode		Lower anode		Bottom		Lower cathode		Upper cathode		Unfractionated	
Lot	P	B	P	B	P	B	P	B	P	B	P	B
Turbidimetric analysis, mg. heat-coagulable protein per ml.	0.03 ±	0.6 ±	0.12	2.4	0.71	*	0.53	*	0.24	2.4	0.48	4.8
Skin test doses per ml., in 100,000,000's	0	0	0.1	0.2	0.5	*	0.5	*	0.25	4.0		
Acid-precipitable protein (qualitative)		0		+++		*		*		0		++++
Refractometric estimate,† mg. per ml.												
Constituent I	0.3	1.3	0.3	1.3	0.3	1.3	Trace	Trace			0.3	1.3
" II		0.4		2.8		2.8		2.2				2.8
" II } III	0.1		0.5		0.7		0.5		0.1		0.7	
" III				0.9		1.6		1.6		0.4		1.6
" IV			Trace	Trace	1.6	1.8	1.6	1.8	1.6	1.8	1.6	1.8
Total	0.4	1.7	0.8	5.0	2.6	7.5	2.1	5.6	1.7	2.2	2.6	7.5

* Not submitted for turbidimetric analysis and skin testing.

† Calculated from the schlieren scanning diagrams on the assumption that the specific refractive increment of each constituent is 0.0018.

tration the fractionation of Lot P could not be so carefully controlled as the fractionation of Lot B; the estimated final positions of the boundaries may have been in error to some extent, because optical observations were possible only during the first third of the experiment.

The turbidimetric data, on the other hand, indicated that Constituent IV was not the only heat-coagulable substance present in Toxin CI; to account for the distribution of heat-coagulable material it was necessary to assume that Constituent II was also coagulated by heat. Fractions containing Constituent II were then found to contain an acid-precipitable protein (see Table III), and Constituent II was identified as an acid-precipitable, heat-coagulable, nucleoprotein-like substance. Our data indicate that this substance has no high erythrogenic activity such as is shown by Constituent IV.

Constituent I was shown not to be heat-coagulable. The data do not exclude the possibility that Constituent III may be coagulated by heat.

Electrophoretic Isolation of Scarlet Fever Toxin—Once the toxic activity had been identified with the slowest migrating constituent (Constituent IV), it was a simple matter to remove the inactive constituents by means of electrophoresis; this was done with Lots A, B, and C.

After removal of all impurities except the small amounts of Constituent III which were not removed by the preliminary fractionation, the toxic Constituent IV appeared to be extremely sensitive to surface denaturation, and the syringe normally used for filling and emptying the electrophoresis cell had to be replaced by a long capillary pipette to avoid denaturation around the minute bubbles of air which were drawn into the solution at the junction of the glass syringe with the needle. After drying by evaporation from the frozen state for storage[2] or concentration it failed to dissolve completely but gave solutions which remained slightly turbid;[3] the unfractionated Toxin CI, on the other hand,

[2] After the preliminary fractionation of Lots A and B the investigation was interrupted temporarily because of the moving of the laboratory from Philadelphia to Newark, Delaware. All the fractions were dried by evaporation from the frozen state to minimize the chance of deterioration during transfer.

[3] Turbid solutions were cleared by centrifuging in the multispeed head, as described earlier.

could be dried and redissolved without visible deterioration. It is believed that this turbidity was the result of surface denaturation which occurred around small bubbles of air trapped in the solid at the time of solution, and that in the unfractionated solutions the toxin was stabilized against such denaturation by the presence of more strongly surface-active impurities.

All the samples of the partially purified toxic constituent (IV) from Lots A, B, and C were combined and concentrated for a final run to remove the remainder of Constituent III (see Fig. 2 and Table I, Run 11). The electrophoretically purified scarlet fever toxin separated by this run was used for the determination of the

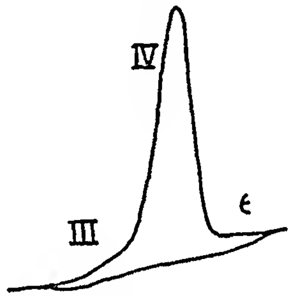

FIG. 2. Final fractionation of scarlet fever toxin. Potential gradient, 4 volts per cm.; time of migration, 2 hours. Descending boundaries, cathode arm. For an explanation of the ε-boundary, see (4). The roman numerals represent constituents.

ultraviolet absorption and the sedimentation velocity in the ultracentrifuge. All of it (including that recovered from the absorption and sedimentation measurements) was then sent by air mail to the Children's Hospital, Pittsburgh, for chemical and clinical tests. It was frozen for shipment to minimize the chances for surface denaturation during transit; it was still frozen when received.

Chemical Properties of Toxic Protein—The erythrogenic protein was not precipitated from Toxin CI by acidification to pH 4 nor by the addition of large volumes of glacial acetic acid, but it was precipitated by tannic acid and trichloroacetic acid. The precipitates could be redissolved by cautious addition of dilute NaOH; the addition of acetic acid to these solutions, however, caused reprecipitation of the protein, thereby indicating denaturation

by the original precipitating acids.[4] Reinecke acid also formed a precipitate, but Reinecke salt did not. Clupein at various pH values did not precipitate the toxin, although as already indicated it removed the acid-precipitable nucleoprotein-like substance.

Emulsification with butyl alcohol and chloroform did not remove the heat-coagulable protein from Toxin CI. This is surprising in view of the sensitivity of the electrophoretically purified toxin to surface denaturation, but it is consistent with the stability of the toxin toward surface denaturation in the presence of the rapidly migrating constituents of the unfractionated Toxin CI.

The toxic protein was completely adsorbed from Toxin CI by a simple aluminum hydroxide gel; this observation coincides with the results of Farago (5), who worked with less pure scarlet fever toxin.

Attempts were made to find a flocculation end-point with Toxin CI and commercial scarlet fever antitoxin as indicated by Rane and Wyman (7) and Pappenheimer.[5] A slightly positive precipitin reaction was obtained over a wide range of protein concentrations, but no definite flocculation point was found. This finding agrees with the work of Bunney and Koerber (8).

Chemical analyses were made both on samples of erythrogenic toxin which had been separated from Toxin CI by precipitation with trichloroacetic acid and on the electrophoretically purified toxin;[6] the results are listed in Table IV.

In general the analyses on the trichloroacetic acid-precipitated toxin agree with those on the electrophoretically purified toxin. The chief inconsistency is the positive phosphorus value for the former, possibly the result of precipitation of the nucleoprotein-like substance (Constituent III) with the toxin.

Sample 4 of electrophoretically purified scarlet fever toxin was estimated refractometrically to contain 10 mg. per ml.; by turbidimetric analysis the concentration was found to be 9 mg. per ml.

[4] Tannic acid has been used by Veldee (6) as a precipitant for the purification and concentration of the erythrogenic toxin.

[5] Pappenheimer, A. M., Jr., personal communication.

[6] Before the electrophoretically purified toxin was received from The Biochemical Research Foundation, one of the authors (A. H. S.) was called into active service in the United States Army Medical Corps. The tests on this toxin were performed by Dr. Marie Andersch of the Children's Hospital, Pittsburgh.

Skin tests on human beings indicated an activity of between 100 and 150 million skin test doses per mg. 3 months later another sample (No. 2), which had been used for the determination of the ultraviolet absorption spectrum, gave an erythrogenic titer of 50 million skin test doses per mg. Both values are lower than the activity of 200 million skin test doses per mg. of heat-coagulable protein which was found in the unfractionated preparations.

TABLE IV

Analysis of Scarlet Fever Toxin

	Electrophoretically purified toxin, Sample 4	Toxin precipitated by trichloroacetic acid
Skin test doses per mg., *millions*	100–150	200
Total nitrogen, %	15.2*	15.1*
Phosphorus, %	Negative†	0.005†
Sulfur, %		0.71‡
Amino nitrogen, ninhydrin reaction	Positive	
Total carbohydrate, Bial's orcinol	Negative	
Carbohydrate, Molisch test	Faintly positive	Faintly positive
Hexosamine	Negative§	
Mucic acid	"	
Biuret test	Positive (lavender)	Positive
Tryptophane, Hopkins-Cole test	Very faintly positive	
Tyrosine, tryptophane	Positive	

* Micro-Dumas (Pregl).

† Method of Berenblum and Chain (9).

‡ Analysis by a micromethod adapted by Dr. Kirner and Dr. Smith of the Carnegie Institute of Technology, Pittsburgh, Pennsylvania (10).

§ Small sample, no hydrolysis.

Some loss of activity may have been caused by the manipulations performed on the toxin during the course of the investigation, particularly the short exposure (4 minutes) of Sample 2 to ultraviolet light. With this exception, however, all procedures were sufficiently mild to preclude denaturation unless the substance was inherently unstable. It is generally recognized that the more highly an enzyme or a toxin is purified, the more unstable it is apt to be. The sensitivity of the electrophoretically purified scarlet fever toxin to surface denaturation has already been mentioned.

Purified diphtheria toxin showed similar behavior (11); dilute unbuffered solutions were partially inactivated by shaking, and even in a properly buffered diluent the purest fractions were found to have lost as much as 25 per cent of their activity.

The non-coagulable portion of Toxin *CI* gave a nitrogen value of 13.5 per cent, a weakly positive biuret reaction, and a slightly positive Molisch reaction. A sample of electrophoretically separated Constituent I containing about 0.5 mg. per ml.[7] formed no precipitate with either sulfosalicylic acid or trichloroacetic acid and gave negative biuret and xanthoproteic reactions; after concentration of the solution by evaporation the biuret reaction was still negative, but the xanthoproteic reaction was faintly positive. Addition of Nessler's reagent after digestion with sulfuric acid indicated a fairly high nitrogen content. The solution gave a negative phloroglucinol reaction and an inconclusive phenylhydrazine reaction, but immediately reduced copper sulfate and potassium permanganate. From these tests, together with the failure to coagulate on heating, it appears that Constituent I may be a polysaccharide.

Electrophoretic Mobility and Homogeneity—At pH 7 the electrophoretically purified toxin had a mobility of -1.93×10^{-5}. It appeared to be substantially homogeneous with respect to charge. The boundary spreading (per cm. of migration) was about the same as that of a sample of serum albumin of similar concentration which had been isolated from horse serum by means of electrophoresis. However, part of the original sharpness of the serum albumin boundaries could be regained by reversing the current, an indication that part of the spreading was due to the progressive separation of constituents which differed very slightly in their rates of migration (12–14). It is possible, therefore, that the electrophoretically purified scarlet fever toxin (Constituent IV) may not be completely homogeneous with respect to electrical migration, and may consist of two or more constituents which differ only very slightly in mobility.

Ultraviolet Absorption Spectrum[8]—The ultraviolet absorption

[7] The chemical tests on the sample of Constituent I were made by Dr. Alfred Bloch of the Microchemical Department of The Biochemical Research Foundation.

[8] The ultraviolet absorption spectrum was determined by Miss Rachel Franklin of The Biochemical Research Foundation.

spectrum of the electrophoretically purified scarlet fever toxin, completely free from pigment, is shown in Fig. 3; the solution contained approximately 3.5 mg. of toxin per ml. The determination was made with a Hilger type E-4 quartz spectrograph and a hydrogen arc, with Hilger echelon cells (15–17). The spectrum contains the characteristic protein absorption band in the near ultraviolet, which demonstrates the presence in the molecule of one or more of the aromatic amino acids, tyrosine, tryptophane, phenylalanine, and histidine. The presence of tyrosine and tryptophane was corroborated by chemical tests (see Table IV).

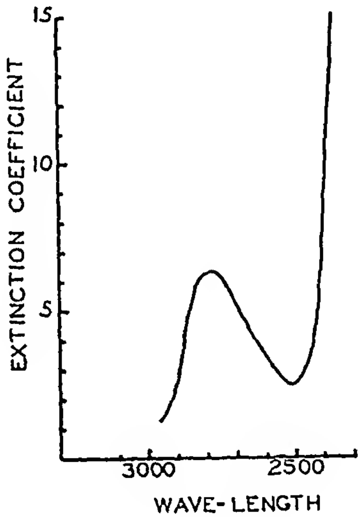

Fig. 3. Ultraviolet absorption spectrum of electrophoretically purified scarlet fever toxin.

Ultracentrifugal Behavior of the Toxin[9]—Sedimentation measurements were made on a solution containing 10 mg. of the toxin per ml. of solution with a Svedberg oil turbine ultracentrifuge ((18) pp. 100, 187) at a cell temperature of 27.0° ± 0.3° and a constant speed of 57,500 R.P.M. (corresponding to a centrifugal force of 240,000 × gravity at the center of the cell). A scale projection apparatus was employed in using the Lamm refractive index method ((18) pp. 253, 287) to follow boundary movement during centrifuging. One sedimentation curve is shown in Fig. 4.

[9] The authors are greatly indebted to E. I. du Pont de Nemours and Company, Inc., for permission to use the oil turbine ultracentrifuge.

From the boundary movement recorded by the curves the sedimentation constant of the toxin, corrected to a basis of sedimentation in water at 20°, was calculated to be $s_{20} = 2.7_4 \times 10^{-13}$ cm. per second per dyne according to the usual equation ((18) Equation 63).[10] For this calculation the partial specific volume of the toxin at 20° was assumed equal to that of diphtheria toxin (V_{20} = 0.736) (19), since insufficient electrophoretically purified scarlet

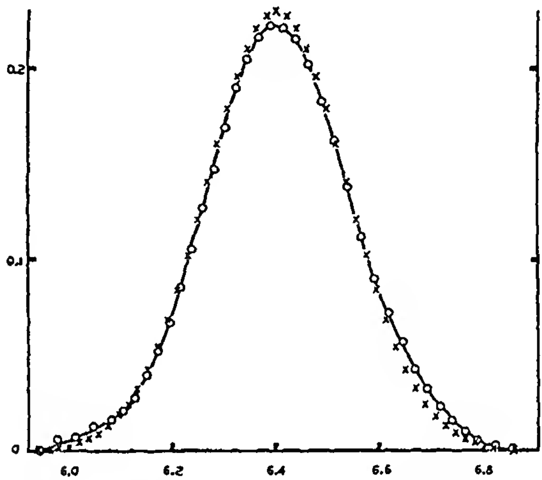

FIG. 4. Sedimentation diagram and theoretical diffusion curve after centrifuging for 141 minutes. O, sedimentation curve; ×, calculated diffusion curve. Abscissa, distance from center of rotation in cm.; ordinate, scale line displacement in mm.

fever toxin was available for a direct determination of the partial specific volume to be made.

The diffusion constant of the toxin was calculated by two methods, first with the half widths (u) of the sedimentation curves at the inflection points and the equation $Dt = u^2F^2/2$ ((18) p. 298, Method III; (20) Equation 40) and secondly with the half widths (x_Δ) of the intercepts made on the x axis by the tangents through the inflection points of the curves and the equation $Dt = x_\Delta^2F^2/8$ ((20) Equation 43). The values of $u^2F^2/2$ and of $x_\Delta^2F^2/8$ thus

[10] For the meanings of the symbols in this and subsequent equations see Svedberg and Pedersen ((18) p. 273 and other corresponding pages cited).

obtained were plotted separately against time of centrifuging (time from the application of the final driving pressure), and the corresponding diffusion constants obtained by determining the slope of the lines through the plotted points (see Fig. 5).

The average of the two values of D thus obtained (*i.e.* $(10.81 + 10.78)/2 = 10.8$) was corrected to 20° and to the viscosity of water ((18) Equation 176), giving $D_{20} = 9.46 \times 10^{-7}$ cm.² per second. While this value of D_{20} is not so precise as one obtained by the normal method of measuring diffusion constants, namely by a

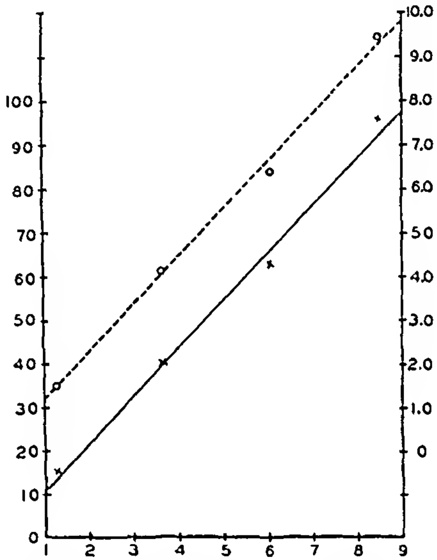

FIG. 5. Relationship between diffusion and time. ×, values of $u^2F^2/2$ (left ordinate); ○, values of $x_\Delta^2F^2/8$ (right ordinate). The abscissa measures the time from the "start" in seconds × 10^{-3}.

separate experiment in a vibration-free diffusion cell with thermostat control, it is adequate for the estimation of homogeneity. Insufficient toxin solution was available for a determination of the diffusion constant with a diffusion cell.

Employing this value for the diffusion constant together with the value of s_{20}, calculations for the molecular weight, M, and the frictional ratio, f/f_0, for the toxin ((18) Equations 67 and 70b respectively) were made. The results obtained were $M = 26{,}700$ and $f/f_0 = 1.1$.

The principal uncertainty in the molecular weight value is due

to the uncertainty in V. In all probability, V does not exceed 0.75, the value for most proteins, nor is it likely to be less than 0.70 ((18) Table 48). $V = 0.75$ leads to a molecular weight of 28,000, $V = 0.70$ to a molecular weight of 23,500.

On the other hand the molecular weight of a scarlet fever toxin, prepared according to the method of Dick and Boor (21), was estimated by Barron, Dick, and Lyman (22) by means of ultrafiltration experiments to be less than that of cytochrome c (13,000) and perhaps more than that of clupein (2000 to 4000).

The homogeneity of the toxin preparation was tested by determining the theoretical diffusion curve ((18) Equation 188) for 141 minutes and superimposing it on the sedimentation diagram obtained after 141 minutes of centrifuging (see Fig. 4). For the calculation the area in sq. cm., defined by the sedimentation curve and the base-line, was obtained by direct measurement from the grapb (Fig. 4), and the value of D_{20} used was the average value (obtained as described previously), not the value referring only to the 141 minute sedimentation curve.

The good fit of the calculated diffusion curve and the 141 minute sedimentation curve over the entire range except at the top indicates a high degree of homogeneity for the toxin, since the agreement of the two curves shows the spreading of the sedimentation curves to be due to diffusion only and not to inhomogeneity of the toxin preparation. The theoretical curve is higher than the observed curve, but this is not a reflection of inhomogeneity, since inhomogeneity would lead to a theoretical curve (of equal area) lower than the obtained curve. The discrepancy at the peak of the two curves is probably due to the fact that the theoretical curve is calculated from the over-all diffusion constant instead of the specific value for this sedimentation curve. In addition, the actual time of diffusion is somewhat greater than the 141 minutes (from the time of application of the final driving pressure) used in calculating the theoretical curve, since some sedimentation and, therefore, also diffusion, occurs while the rotor is being brought up to high speed. Though these factors cause some discrepancy between the heights of the two curves, the average value of the diffusion constant as calculated is not affected by this uncertainty, since it has been calculated only from observations made when the rotor was travelling at top speed.

For purposes of comparison with other toxins the values of the constants of the electrophoretically purified scarlet fever toxin are listed in Table V. It will be seen that the molecular weight, 27,000, of the scarlet fever toxin is of the same order as that of crotoxin. Further, the frictional ratio of the toxin is practically identical with that for many proteins, and its value of 1.1 shows it to be essentially spherical and probably somewhat hydrated in solution.

TABLE V

Molecular Constants of Some Toxic Proteins

	Scarlet fever toxin	Diphtheria* toxin	Human tubercle* bacillus protein	Crotoxin*
Sedimentation constant........	2.7×10^{-13}	4.6×10^{-13}	3.3×10^{-13}	3.13×10^{-13}
Diffusion constant	9.5×10^{-7}	6.0×10^{-7}	8.2×10^{-7}	8.59×10^{-7}
Mol. wt...........	27,000	74,000	32,000	30,000
Frictional ratio...	1.1	1.22		1.2
Specific volume...	0.736†	0.736	0.7	0.704

* References; diphtheria toxin (19), human tubercle bacillus protein (23), crotoxin (24).

† Assumed.

DISCUSSION

Of the electrophoretically distinct constituents present in the chemically isolated scarlet fever toxin (Toxin CI), only the slowest migrating (Constituent IV) possessed toxic activity. Its high degree of homogeneity with respect to both charge and molecular weight, and in particular its high toxic activity, are fairly strong indications that it actually is identical with scarlet fever (erythrogenic) toxin.

The electrophoretically purified scarlet fever toxin displayed somewhat reduced activity per mg. This may be ascribed to spontaneous inactivation following removal of stabilizing impurities. The diminished activity suggests that some inactive toxin may have been present in the solutions used for the final electrophoresis experiment and for the determination of the sedimentation velocity in the ultracentrifuge. The electrophoresis and sedimentation diagrams, however, gave no evidence of hetero-

geneity, and it seems safe to conclude that spontaneous inactivation does not appreciably change either the mobility or the molecular weight of the scarlet fever toxin.

Diphtheria toxin resembles scarlet fever toxin with respect to instability and inactivation. Highly purified diphtheria toxin has also been found to possess diminished activity; like electrophoretically purified scarlet fever toxin, it is sensitive to surface denaturation; and it appears to be homogeneous with respect to both sedimentation and electrophoresis in spite of partial loss of activity (25).

SUMMARY

Chemically isolated scarlet fever (erythrogenic) toxin was analyzed by electrophoresis in the Tiselius apparatus and was found to contain five electrophoretically distinct constituents. The slowest migrating constituent was identified as the erythrogenic toxic constituent, and was isolated by electrophoresis.

The electrophoretically separated scarlet fever toxin was shown by chemical tests, by its property of heat coagulability, and by its ultraviolet absorption spectrum to be a protein. The electrophoretic mobility was found to be -1.93×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ at pH 7 in the buffer used, the sedimentation constant 2.7×10^{-13} cm. sec.$^{-1}$ dyne^{-1}, the diffusion constant 9.5×10^{-7} cm.^{2} sec.$^{-1}$, the molecular weight 27,000, and the frictional ratio 1.1. The high toxic activity of the electrophoretically separated toxin (100 to 150 million skin test doses per mg.) and its substantial homogeneity with respect to both electrophoresis and sedimentation are evidence that this material is probably pure scarlet fever (erythrogenic) toxin.

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THE HYDROGEN ION DISSOCIATION CURVE OF β-LACTOGLOBULIN

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A few years ago, Palmer (1) isolated a crystalline globulin from the albumin fraction of cow's milk. This protein has been variously designated in the literature. We are now suggesting that it be named β-lactoglobulin. The Greek prefix derives from a publication of Pedersen (2) who distinguished three major components of skim milk on the basis of ultracentrifugal studies. One of these, which he called the β component, was shown to have the same sedimentation constant as the crystalline preparation of Palmer. The name, β-lactoglobulin, serves to relate the purified protein to a definite component of milk serum, to emphasize its globulin character, and to distinguish it from the classical globulin fraction of milk.

On the basis of ultracentrifugal and electrophoretic studies, Pedersen (3) concluded that β-lactoglobulin behaves in solution, between pH 1 and 9, as "a monodisperse protein with a molecular weight of 39,000 and an isoelectric point of 5.19 (acetate buffers)." Recently the dielectric constants of solutions of β-lactoglobulin have been measured (4). The results indicate that the electric moment of the molecule is unusually large. This conclusion invests with a particular interest the problem of the number, nature, and distribution of the ionizing groups.

In the present paper we are reporting the results of a series of studies of the hydrogen ion dissociation curve of β-lactoglobulin. The variables whose effects upon the curve have been examined are the concentration of protein, the concentration of KCl, the temperature, and the addition of formaldehyde. From the results

obtained an estimate has been made of the number and nature of the ionizing groups which contribute to the curve. This estimate is compared with the results of preliminary analyses of the number of acidic and basic amino acids present in the hydrolysis products of β-lactoglobulin. The paper concludes with a theoretical analysis of the shape of the dissociation curve in terms of an electrostatic model first outlined by Linderstrøm-Lang (5) and a consideration of the relation of the net charge to the electrophoretic mobility.

Methods

The protein was prepared by the method of Palmer and crystallized three or more times. Stock solutions were made by dissolving the crystals in water with the aid of a minimum amount of standard NaOH. The dry weight, ash, and nitrogen of these solutions were determined. The pH was always close to 6.0. Reaction mixtures were then prepared by the addition of measured volumes of standard KCl and of standard HCl or NaOH to a measured volume of stock solution, followed by dilution to a predetermined concentration of protein and of electrolyte. To compensate for the variable additions of acid and base, the amounts of KCl added were so adjusted that the [KCl] + [HCl] or [KCl] + [NaOH] of any one curve remained constant. This method was adopted as the simplest experimental approximation to a series of mixtures of constant ionic strength.

In the work on the effect of [KCl], the hydrogen electrode potential of each reaction mixture was determined in a Clark rocking electrode at 25°, equipped with a saturated calomel half-cell and a saturated KCl bridge. The reference electrode was calibrated with 0.01 M HCl in 0.09 M KCl, to which solution we assigned a pH of 2.10 and a potential at the junction with saturated KCl of −2.2 millivolts (6). The liquid junction potentials of the reaction mixtures were calculated from Henderson's equation (Table I), the value adopted for the transference number of K^+ in KCl being 0.490 (7).

The effects of [formaldehyde] were studied both with the hydrogen electrode and with a glass electrode-vacuum tube assembly. The glass electrode behaves more consistently in the presence of

formaldehyde than does the hydrogen electrode. The observations on the effects of temperature and of [protein] were made with the glass electrode only. It was calibrated at each temperature in the manner described by Wyman (8).

The curves which will be discussed represent the relation between pH and a quantity designated as h. The latter is defined as the number of hydrogen ions combined with (+) or dissociated from (−) 1 mole of isoionic protein. We have adopted a value of 40,000 (3) for the molecular weight of β-lactoglobulin. Assuming, now, that combination of the protein with ions other than H^+ and

TABLE I

[KCl]	Calculated liquid junction potential	$-\mathrm{Log}\ \gamma_{H^+}$	$-\mathrm{Log}\ \frac{K_w}{\gamma_{OH^-}}$
M	*mv.*		
2.1	−0.3	−0.06	13.87
0.670	−0.9	+0.115	13.84
0.270	−1.4	0.125	13.85
0.135	−1.8	0.113	13.87
0.069	−2.1	0.095	13.90
0.035	−2.5	0.083	13.92
0.019	−2.9	0.072	13.94
0.010	−3.2	0.060	13.96

OH^- is relatively insignificant, h is equal to the net charge and is given by

$$\frac{h \cdot g}{40{,}000} = [Cl^-] + [OH^-] - [K^+] - [Na^+] - [H^+] \qquad (1)$$

where each term on the right-hand side is a molal concentration and g is the weight in gm. of dry ash-free protein in 1000 gm. of water. We have calculated $[H^+]$ and $[OH^-]$ from the assumptions that

$$\mathrm{pH} = -\log [H^+]\gamma_{H^+} = \log [OH^-] \frac{\gamma_{OH^-}}{K_w}$$

The values assigned to the two activity coefficients involved are given in Table I. They were calculated from our own observations of the pH of solutions of HCl or of NaOH (0.001 to 0.01 M) having

the same concentrations of KCl as the various protein reaction mixtures. The known compositions of the latter fix the remaining quantities in Equation 1 and permit the calculation of h.

Reversibility—The question of the extent to which the curves represent reversible equilibria is important. We have used two criteria: (*a*) the stability of the pH of a reaction mixture for several hours, and (*b*) the precision with which a reaction mixture returned to the isoelectric point when the HCl or NaOH originally added was quantitatively neutralized. Applying these tests to a representative range of mixtures, we have satisfied ourselves that the reaction with H^+ is reversible between pH 2 and 10 in the absence of formaldehyde. In the neighborhood of pH 11 there are indications of irreversible reactions which become very rapid at greater alkalinity. At pH 12, β-lactoglobulin is converted, in less than 5 minutes, to a product insoluble in dilute salt solutions in the isoelectric region. The dissociation curve of this "metaprotein" differs significantly from that of the unaltered protein. It is interesting to note that Pedersen infers from the behavior of β-lactoglobulin in the ultracentrifuge that the protein suffers an irreversible breakdown above pH 11.

Dissociation Curves

Isoionic Point—All preparations of the protein which we have tested have given a pH of 5.18 to 5.20 when dissolved in dilute NaCl or KCl, irrespective of the concentration of protein. Moreover the addition of KCl up to 2 M did not significantly change this pH. If we are justified in identifying as the isoionic point that point on the dissociation curve which is independent of μ, we may conclude that the protein crystals are in the isoionic condition. This is, perhaps, to be expected from the method of preparation. It is interesting to observe that β-lactoglobulin exhibits an isoionic point which is identical with the electrophoretic isoelectric point in 0.02 M acetate. It would seem probable that the isoelectric point of β-lactoglobulin, unlike that of some proteins, will be found to be substantially independent of ionic strength.

Effect of [KCl]—Some forty or more independent reaction mixtures have been measured at each experimental ionic strength. In the majority of these the [protein] was close to 0.5 per cent. For the sake of greater precision in the calculation of h at the ex-

tremities of the curves the [protein] was raised to 1, 2, or 4 per cent at the lower and higher limits of pH. The observations have accumulated over a period of years and relate to three different preparations of protein and to two different electrode assemblies.

TABLE II

Effect of Ionic Strength (KCl) on Dissociation of β-Lactoglobulin

h	0.010 μ	0.019 μ	0.035 μ	0.069 μ	0.135 μ	0.270 μ	0.67 μ	2.1 μ
	pH							
45							1.90	2.02
44						2.13	2.30	2.42
42				2.10	2.25	2.45	2.70	2.95
40			2.12	2.35	2.53	2.70	2.95	3.16
35		2.36	2.62	2.82	2.96	3.14	3.35	3.52
30	2.65	2.83	3.00	3.19	3.32	3.45	3.67	3.80
25	3.08	3.23	3.36	3.53	3.64	3.74	3.92	4.03
20	3.46	3.60	3.72	3.84	3.93	4.02	4.16	4.25
15	3.86	3.96	4.06	4.15	4.22	4.30	4.40	4.47
10	4.28	4.35	4.42	4.48	4.52	4.57	4.64	4.69
5	4.73	4.75	4.78	4.82	4.85	4.87	4.89	4.92
0	5.18	5.18	5.18	5.18	5.18	5.18	5.18	5.18
−5	5.75	5.72	5.69	5.64	5.63	5.60	5.54	5.51
−10	6.55	6.50	6.45	6.38	6.35	6.30	6.25	6.20
−15	7.55	7.45	7.35	7.28	7.23	7.16	7.10	7.00
−20		8.80	8.65	8.55	8.45	8.40	8.35	8.30
−25			9.72	9.60	9.48	9.40	9.40	9.50
−30			10.20	10.10	10.05	10.02	9.90	10.0
−35			10.60	10.47	10.35	10.30	10.30	
−40			10.83	10.70	10.60	10.55	10.50	
−45			11.05	10.93	10.80	10.75	10.75	
	Values of w							
Theory.....	0.064	0.055	0.047	0.0395	0.0315	0.026	0.020	0.015
Observed...	0.061	0.052	0.046	0.038	0.032	0.027	0.020	0.015

The results are summarized in Table II. The values of h recorded were obtained by graphic interpolation in smooth curves drawn through the experimental points. The great majority of the observations falls within 0.2 equivalent of their curve as defined by Table II. No reproducible observation within the range of pH 2.5 to 10 falls more than 0.5 equivalent from the curve.

Effect of [Protein]—Dissociation curves (pH 2.5 to 7) for four concentrations of protein (0.44, 0.88, 1.77, and 3.54 gm. per 100 ml.) were first determined in the presence of 0.27 M KCl. The four curves were indistinguishable from one another and from the curve for 0.27 μ in Table II. At this [KCl] the contribution of protein to the ionic strength is, presumably, negligible at any pH. Consequently, the identity of the curves is to be expected if the only anticipated effect of varying the concentration of protein is a change in the ionic strength of its ions. In the case of β-lactoglobulin, however, another influence must be considered. The dielectric constant increment of this protein is reported (4) to be about 1.4 per gm. per kilo of water. This corresponds to dielectric constants of 85, 91, 104, and 128 for the four solutions which we titrated. It would be surprising if such large changes in dielectric constant were not reflected in pronounced displacements of the dissociation curves. We, therefore, conclude that the dielectric constants which have been measured are not those which determine the magnitude of the electrostatic forces involved in the dissociation of the protein.

Experiments were then conducted in the absence of KCl with concentrations of protein varying from 0.44 to 4.91 gm. per 100 ml. In these, definite, though small, effects of [protein] were evident. Data for four curves are presented in Table III which was compiled in the same manner as was Table II. The general effect of increasing [protein] in the absence of KCl is to displace the curve in the same direction as does an increase of [KCl] in the presence of a constant, but low, concentration of protein.

Effect of Temperature—Curves (pH 4 to 9) in 0.27 M KCl have been determined at 7°, 27°, and 43°. Wyman (8) has made the assumption that the apparent heats of dissociation (Q') of the protein at chosen values of h may be calculated from the approximation

$$Q' = -4.79 \frac{T_1 T_2}{T_2 - T_1} \cdot (\mathrm{pH}_2 - \mathrm{pH}_1)$$

where pH_1 and pH_2 represent the observed values of pH corresponding to a particular value of h at the two temperatures, T_1 and T_2. We have applied this equation to our observations. The results of three experiments are shown in Fig. 1.

Effect of Formaldehyde—Upon addition of formaldehyde to a solution of the protein which is alkaline to the isoelectric point,

TABLE III
Effect of Protein Concentration

h	pH			
	Gm. protein per kilo water			
	8.8	24.5	35.1	49.1
40		2.24		2.35
35	2.45	2.63	2.70	2.78
30	2.75	2.95	3.02	3.10
25	3.10	3.25	3.35	3.42
20	3.43	3.56	3.65	3.70
15	3.75	3.88	3.95	3.98
10	4.17	4.25	4.30	4.33
5	4.67	4.70	4.72	4.74
0	5.19	5.19	5.19	5.19
−5	5.84	5.83	5.82	5.78
−10	6.70	6.66	6.64	6.60

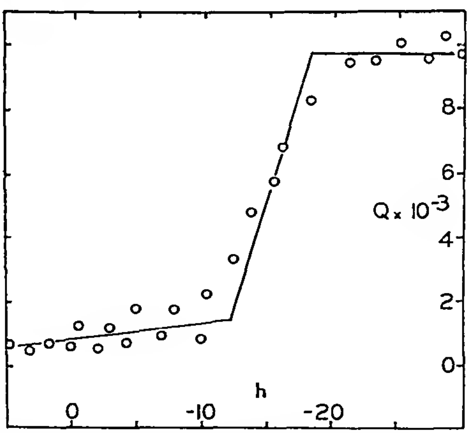

FIG. 1. Relation of the apparent heat of dissociation (Q') to the net charge (h). $\mu = 0.27$, $T_1 = 7°$, $T_2 = 27°$.

there is an abrupt fall in pH to a relatively stable value. In restricted regions of pH, however, this large initial change is followed by a small protracted fall in pH which may continue for

many hours. We assume that it is the initial liberation of H^+ which corresponds with the reversible reaction of amino groups. The observations upon which our curves have been based were those recorded within 5 to 20 minutes of the addition of formaldehyde. The results are shown in Fig. 2. The calculations were made after the manner of Kekwick and Cannan (9).

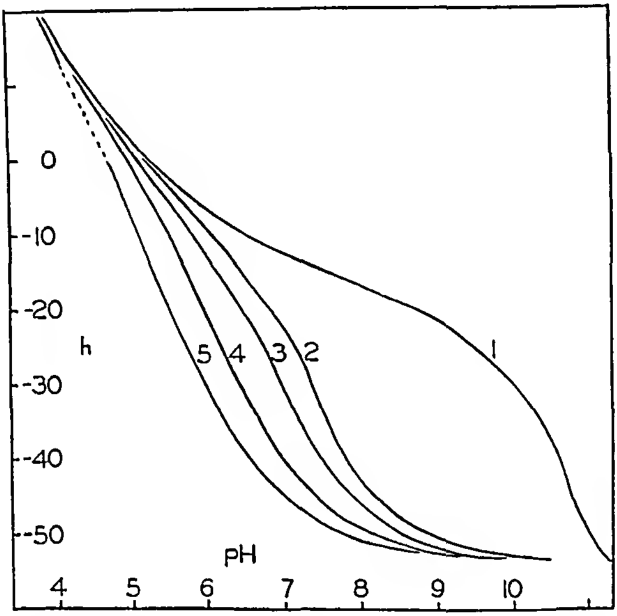

FIG. 2. Dissociation curves in solutions of formaldehyde. Curve 1, without formaldehyde; Curve 2, 0.13 M formaldehyde; Curve 3, 0.33 M formaldehyde; Curve 4, 1.00 M formaldehyde; Curve 5, 3.00 M formaldehyde.

Analytical

No amino acid analyses of β-lactoglobulin have yet been published. The determinations of chief interest in the present connection are those of the basic and of the dicarboxylic acids together with those of the amide and of the free amino groups.

Amino Groups—We have made determinations of the amino groups which react with nitrous acid under the conditions of Van Slyke's manometric method. With the object of disclosing secondary reactions, the time of reaction was varied from 5 to 90 minutes, with the results shown in Table IV. The primary reaction seems to be substantially complete in 30 minutes and corre-

sponds with the presence of 35 groups. Miller (10), using a reaction time of 30 minutes, reports 34 groups on the basis of a molecular weight of 39,000.

Basic Amino Acids—A series of determinations has been made by a modification of the methods of Block (11) and Tristam (12) in which lysine was isolated as the picrate, arginine as the flavianate, and histidine as the nitranilate. The amounts of protein used for each analysis were 3 to 5 gm. The mean results correspond with the isolation of 6 moles of histidine, 6.4 of arginine, and 29.1 of lysine per 40,000 gm. Dr. H. B. Vickery has kindly made for us two determinations of arginine by his latest method (13). His results correspond with 6.1 moles of arginine. We tentatively

TABLE IV

Amino Groups of β-Lactoglobulin; Van Slyke Manometric Method; 22–23°

Reaction time	NH_2	
	$\times 10^3$ per gm.	Per 40,000 gm.
min.	*equivalent*	*equivalents*
5	0.637	25.5
15	0.838	33.5
30	0.880	35.2
60	0.896	35.8
90	0.902	36.1

The observations are the mean of three concordant determinations.

conclude that β-lactoglobulin contains 6 arginine, 6 histidine, and 29 lysine residues.

Dicarboxylic Acids—We have not had an opportunity to attempt a quantitative isolation of glutamic and aspartic acids. By an indirect method, details of which will be published elsewhere, we have, however, sought to obtain an approximate value for the total dicarboxylic acids. The method depends upon a differential electrometric titration of the barium salts precipitated from the protein hydrolysate by alcohol. The results are consistent with the presence of 75 to 80 moles of dicarboxylic acids.

Amide Groups—The amount of ammonia formed during the acid hydrolysis of β-lactoglobulin depends upon the conditions of hydrolysis. Two stages in the reaction may be distinguished.

There is an early rapid evolution of ammonia followed by a further slow, sustained production. The latter follows a roughly linear course for long periods. We assume that it is the first stage, which corresponds with the hydrolysis of amides. A linear extrapolation to zero time of the secondary phase of the reaction in M HCl at 100° gave 30.3 amide groups. This result was confirmed by an experiment in which 0.25 M HCl at 85° was used. Under these conditions the rate of the secondary reaction was negligible and the reaction curve gave a satisfactory first order constant of 0.098 (common logarithms, hours) for $a = 30$.

TABLE V

Maximum Acid-Binding Capacity

h = equivalents per 40,000 gm.

[KCl] 0.67 M [Protein] 3.3 per cent		[KCl] 0.27 M [Protein] 7 per cent	
pH	h	pH	h
2.13	44.5	2.11	44.0
1.95	45.0	1.97	44.8
1.82	45.4	1.82	45.5
1.73	45.5	1.68	46.0
1.66	45.8	1.54	45.7
1.55	45.6	1.47	46.1

DISCUSSION

Stoichiometry—At low pH the various KCl curves approach a common maximum value of h. In order to determine this maximum with precision we have made a series of observations below pH 2 on solutions containing high concentrations of both protein and KCl. The results of two experiments are given in Table V. A survey of all the experiments leads to the choice of 46 as the most probable value for the maximum cation charge.

At high pH the curves suggest an approach to a maximum anion charge. It is not, however, possible to establish the value of this maximum, as it is not yet fully attained at the extreme alkalinities at which accurate values of h may be calculated. A plausible extrapolation would suggest a maximum base-binding capacity of 60 to 65. The significance of even so broad an approximation is,

however, uncertain in view of the participation of irreversible reactions above pH 11. The estimates of acid- and base-binding capacities arrived at above materially exceed those reported from the Carlsberg laboratory (14). The latter were based upon colorimetric titrations, the significance of which, when applied to proteins, is uncertain.

A number of proteins, *e.g.* egg albumin, exhibit a well defined stoichiometric point in the region of pH 8.5. In the curves of β-lactoglobulin this is not so well defined, but a careful inspection of the curves indicates a transition point at $h = -18$ to -19. This value of h should correspond with the total COOH groups less the sum of the amino and guanidino cations.

In the presence of formaldehyde a much more clearly defined anion charge of 52 to 53 is attained at the same pH level. Each curve attains this maximum at a pH dependent on the $[CH_2O]$ to which it corresponds, but maintains it for about a full pH unit before reaching a pH beyond which accurate computations of h can no longer be made. Combining the four curves, we thus have a constant anion charge in formaldehyde solution over the pH range of 8.5 to 10.5. It follows that practically no groups, other than amino groups, contribute to the curve in this region. Since the basicity of the guanidino group is not notably weakened by formaldehyde, the anion charge of 52 to 53 should equal the total carboxyls less the guanidino cations.

Wyman (8) has shown how temperature effects may be exploited to arrive at an estimate of the magnitude of the contribution of imidazole groups to a dissociation curve. He assumes that below pH 9.5 only COOH, imidazole, and amino groups are involved and suggests that the heats of ionization of these would be about ± 1000, $+6000$, and $+10,000$ calories respectively. In Fig. 1 we see that Q' is small for values of h positive to -12 and then increases sharply as h becomes more negative to reach a plateau of about 10,000 for values of h negative to -18. This would suggest the presence of not more than 6 imidazole groups.

Summarizing the evidence from titration data only, we have the following: (*a*) total cations = 46, (*b*) COOH − guanidino = 52 to 53, (*c*) COOH − guanidino − amino = 18 to 19, (*d*) imidazole = 6, (*e*) amino = (*b*) − (*c*) = 34 (33 to 35), (*f*) guanidino = (*a*) − (*d*) − (*e*) = 6 (5 to 7), (*g*) carboxyl = (*b*) + (*f*) = 58 (57 to 60).

It will be seen that the number of guanidino and imidazole groups agrees with the content of arginine and of histidine respectively. The carboxyl content, however, exceeds by about 10 the difference between the total number of dicarboxylic acids and the number of amide groups. The number of amino groups disclosed by formaldehyde titration is in agreement with those found to react with nitrous acid. It exceeds by 5, however, the amount of lysine which we have succeeded in recovering as the picrate. This echoes a situation observed in the case of egg albumin (9) and quite recently in cytochrome *c* by Theorell and Åkesson (15).

In the theoretical treatment which follows we will assume that a molecule (40,000 gm.) of β-lactoglobulin contains 58 carboxyl groups, 6 guanidino, 6 imidazole, and 34 amino groups, making a total of 46 cations. We make no assumption respecting the presence of phenolic groups. These would not be expected to dissociate below pH 10 and, therefore, should contribute neither to the curve nor to the net charge below this point. Our analysis of the curves has not been extended beyond pH 10. If we accept as significant the estimated maximum base-binding power of 60 to 65 at or beyond pH 12, then the curve could accommodate 2 to 7 phenolic groups between pH 10 and 12.

Theoretical Dissociation Curves—It is convenient to consider separately the theoretical contributions to the curve of the carboxyl, imidazole, and amino groups. Inasmuch as the guanidino groups are assumed to be strong bases, their only effect on the curve over the experimental pH range will be the contribution of a fixed cation charge to the net charge.

Below pH 6, a typical protein should behave as a polycarboxylic acid having a fixed cation charge, n, equal to the sum of the basic groups and a variable net charge, h, whose magnitude will depend on the number of protons which have dissociated from the m carboxyl groups present. The successive dissociations may be defined by m constants, K'_1, K'_x, K'_m, such that

$$K'_x = \frac{[P_{n-x}]}{[P_{n-x+1}]} \cdot a_{\mathrm{H}}$$

where a_{H} is the hydrogen ion activity and $[P]$ the concentration of the protein ion whose charge is denoted by the subscript. Combining the m equations, we may derive the relation

$$h = \frac{n \cdot a_{\mathrm{H}}^m + (n-1) \cdot a_{\mathrm{H}}^{m-1} K_1' + (n-2) a_{\mathrm{H}}^{m-2} K_1' K_2' + \cdots + (n-m) K_1' K_2' \cdots K_m'}{a_{\mathrm{H}}^m + a_{\mathrm{H}}^{m-1} K_1' + a_{\mathrm{H}}^{m-2} K_1' K_2' + \cdots + K_1' K_2' \cdots K_m'} \tag{2}$$

The carboxyl groups of proteins are believed to be those of the side chains of aspartic and glutamic acids. The intrinsic dissociating tendency of these two types of group may be expected to differ from one another, although we anticipate that in a large peptide this difference will be small. Since we have no information as to the relative numbers of the two types present in a particular protein, we will proceed on the assumption that all of the COOH groups may be described by a single intrinsic dissociation constant, K_0.

In the absence of interaction between the ampholyte ion and the proton or the ion atmosphere, statistical considerations lead to the relation

$$K_x' = K_0 \cdot \frac{m - x + 1}{x} \tag{3}$$

Under these conditions, as von Muralt (16) and others have shown, Equation 2 reduces to

$$a_{\mathrm{H}} = K_0 \cdot \frac{m - (n - h)}{n - h} \tag{4}$$

This is the equation for the dissociation of m equivalents of a *univalent* acid having a dissociation constant K_0 and a cation charge n/m. We will call this the ideal curve of the carboxyl groups of the protein. Now, at constant ionic strength, this section of the experimental curves of a number of proteins does exhibit the general symmetry of the ideal curve, but covers a much wider span of pH. As μ increases, the span contracts and approaches but does not attain that of the ideal. Qualitatively, this is the situation which would be anticipated on accepted principles of electrostatic interaction. The spreading of the curve would be attributed to interaction of the protein charge with the dissociating protons and the contraction, which is observed as μ increases, to interaction of the protein ions with the ion atmosphere.

Linderstrøm-Lang (5) was the first to combine the theories of Bjerrum and of Debye-Hückel in a quantitative description of the

dissociation of a polyvalent ampholyte. His treatment has recently been discussed by Kirkwood (17) and has been applied by us (18) to the dissociation curve of egg albumin. When the argument of Linderstrøm-Lang is applied to the case of an ampholyte having the characteristics defined by Equation 2, there may be derived the relation

$$\mathrm{pK}'_x = \mathrm{pK}^0_0 - 0.868w(n - x + 0.5) - \log \frac{m - x + 1}{x} \tag{5}$$

wherein[1]

$$K^0_0 = K_0 \cdot e^{\pm b},\ w = b\left[1 - \frac{\kappa r}{1 + \kappa a}\right] \quad \text{and} \quad b = \frac{e^2}{2\epsilon rkT} \tag{5, a}$$

In Equation 5, a, κ is the familiar function of the ionic strength in the Debye-Hückel theory and b is the charge interaction term of Bjerrum, while a is the "distance of closest approach" of the ions of the atmosphere to the protein ion and r is the distance separating the proton from the charge on the protein in the theory of Bjerrum. If we assume that the dielectric constant (ϵ) of the protein solution is the same as that of the solvent, then, at 25°, when $\epsilon = 78.8$, $b = (3.538 \times 10^{-8})/r$. Assuming, further, that the protein is a spherical molecule and that the charge distribution is a random one, r may be identified with the radius of the protein molecule. Then $a = r + r'$, where r' is the effective mean radius of the ions surrounding the protein. In solutions of KCl we may assign to r' a value of 2 Å. (19). The value of r, on the other hand, may be expected to exceed 20 Å. In the case of a protein, therefore, the value of b should not exceed 0.17 and that of w should vary with μ only between the extreme limits of 0.17 and 0.

For fixed values of m, n, and K^0_0, any selected value of w may be introduced into Equation 5 to yield the m dissociation constants. With the aid of these, data for the construction of a dissociation curve may be computed from Equation 2. We have examined the characteristics of such theoretical curves for a representative range of values of w and of m. They are all symmetrical about

[1] The sign of the exponent in the term $e^{\pm b}$ is *plus* for the case of a carboxyl or other uncharged acid group and *minus* for an imidazole cation or other positively charged acid group. In proteins the difference between K_0 and K^0_0 is quite small.

their mid-point and all intersect at $h = 0$. The ideal curve is, of course, the curve for $w = 0$. As w assumes increasing positive values, m being maintained constant, the span of the curve on the pH scale increases. Finally the curve begins to assume an undulatory form reflecting the separate contributions of the m dissociations. This, however, only becomes perceptible as w rises above unity, a value greatly in excess of that compatible with the dimensions of protein molecules. Under the restrictions imposed by the latter, the curves are smooth and qualitatively resemble those experimentally derived.

We may proceed to a detailed application of Equations 5 and 2 to an experimental curve in the following manner. (*a*) The values of m and n are obtained from a stoichiometric analysis of the curve. (*b*) If $m > n$, as is the case with many proteins, the isoionic point (pI′) will fall within the carboxyl section of the curve. Since $h = 0$ at this point on all curves, we may derive a value for K_0^0 from the experimental value of pI′ with the aid of Equation 4. (*c*) It is evident, from Equation 5, that the m values of pK'_x are symmetrically distributed about their mean and that the mean is equal to $\mathrm{pK}'_{(m+1)/2}$. It follows that the dissociation curve is also symmetrical about its mid-point and that the pH of the latter ($\mathrm{pH_{mid.}}$) is given by

$$\mathrm{pH_{mid.}} = \mathrm{pK}'_{\frac{m+1}{2}} = \mathrm{pK}_0^0 - 0.868w \cdot \frac{2n - m}{2} \tag{6}$$

K_0^0 being known, w may be computed from the experimental value of $\mathrm{pH_{mid.}}$ (*d*) Alternatively, we may make use of a graphic approximation which was derived by Linderstrøm-Lang. A theoretical curve is approximately linear in the region of the mid-point. Linderstrøm-Lang has shown that this linear slope is given by

$$\frac{\Delta \mathrm{pH}}{\Delta h} = -0.868\left(w + \frac{2}{m}\right) \tag{7}$$

A value for w may thereby be obtained without knowledge of K_0^0. The latter can then be computed from Equation 6.

We have applied this procedure to the appropriate segments of the experimental curves, with the results shown in Table VI. The linear slopes of the curves in the region of pH 4 to 5 yield a series of values of w which, when inserted in Equation 6, do lead to

a consistent value for K_0^0. The latter, moreover, is in agreement with the value computed from pI′ with the aid of Equation 4. This agreement has significance because pI′ is located outside the linear segments of the curves. Finally, we have inserted the above values of w in Equations 5 and 2 and computed the theoretical values of h corresponding to a range of values of pH covering substantially the whole course of each curve (pH 2 to 6). When these were compared with the corresponding values obtained by interpolation in the experimental curves, the agreement was quite satisfactory in every case. One example of this comparison is given in Table VII. We conclude that a single value of w suffices to describe the whole course of the carboxyl contribution to the curve at constant ionic strength.

TABLE VI

Values of K_0^0

Carboxyl groups; m = 58; n = 46					Imidazole groups; m = 6; n = −12	
μ	$-\frac{\Delta pH}{\Delta h}$	$w_{exp.}$ (Equation 7)	$pH_{mid.}$	pK_0^0 (Equation 6)	$pH_{mid.}$	pK_0^0 (Equation 6)
0.010	0.083	0.061	3.70	4.60	7.55	6.76
0.019	0.075	0.052	3.82	4.59	7.45	6.78
0.035	0.070	0.046	3.92	4.60	7.35	6.75
0.069	0.063	0.038	4.03	4.59	7.28	6.78
0.135	0.058	0.032	4.10	4.58	7.23	6.82
0.270	0.0535	0.027	4.19	4.59	7.16	6.82
0.670	0.0475	0.020	4.30	4.60	7.10	6.83
2.10	0.043	0.015	4.38	4.60	7.00	6.80
From pI′ = 5.18 (Equation 4)..........				4.60		

In the case of egg albumin, the values of w derived from the carboxyl segments were successfully applied to the sections of the curves dominated by the imidazole and the amino groups respectively. In the present case, we also obtain a consistent value for K_0^0 (imidazole) from Equation 6, using the values of w derived from the carboxyl sections (Table VI). On the other hand, the midpoints and slopes of the amino segments correspond with much smaller values of w. For this anomaly we have no explanation

except to note that Pedersen has reported an abrupt change in sedimentation constant of β-lactoglobulin above pH 9.

We have still to compare the values of w derived from the experimental curves with those which may be calculated from Equation 5, a. The diffusion constant (D) of β-lactoglobulin may be used to compute a value for r on the assumption that $r = kT/6\pi\eta D$. Pedersen (3) reports three determinations by Polson of D_{20} in the isolectric region. The mean is 7.26×10^{-7} after correction to the basis of diffusion in pure water. This corresponds with $r = 29.1$ Å. The value of a is, therefore, 31.1 Å. The values

TABLE VII

Theoretical Curve for $w = 0.038$ ($\mu = 0.069$) (Equations 5 and 2)

$pK_0^0 = 4.60$; $m = 58$; $n = 46$.

pH	h	
	Theory	Observed
1.5	44.7	
2.0	42.6	42.4
2.5	38.6	38.4
3.0	32.7	32.5
3.5	25.4	25.5
4.0	17.6	17.5
4.5	9.7	9.6
5.0	2.3	2.3
5.5	−3.8	−3.5
6.0	−8.1	−7.8
6.5	−10.5	−10.8

of w computed from Equation 5, a on this basis are compared, in Table II, with those experimentally derived. They differ, in no case, by more than 6 per cent. This highly satisfactory result must, in some degree, be regarded as fortuitous. Several of the assumptions upon which the theory is based, *e.g.* spherical protein ions, a charge distribution having central symmetry, a single K_0^0 for all carboxyl groups, are of very dubious validity. Indeed one disconcerting result is the conclusion that the carboxyl groups of β-lactoglobulin have $pK_0^0 = 4.6$, while the corresponding constant of egg albumin is 4.29. On the other hand, the imidazole constant has been found to be 6.7 to 6.8 in both proteins.

Relation between Net Charge (h) *and Electrophoretic Mobility* (u)—The electrophoretic mobilities of β-lactoglobulin have been determined by Pedersen at 20° and $\mu = 0.02$ over a wide pH range. His results are given in Table VIII, together with the corresponding values of h. The latter were obtained by interpolation in our curve for $\mu = 0.019$, after introduction of a small temperature correction derived from our studies of the effects of temperature on

TABLE VIII

Relation between Mobility (u) *and Net Charge* (h)

20°; $\mu = 0.02$.

pH	Electrophoresis buffer	$u \times 10^5$	h	$\frac{h}{u} \times 10^{-5}$
3.22	Acetate	19.6	25.0	1.27
3.66	"	17.3	19.1	1.10
4.18	"	12.1	12.0	0.99
4.48	"	8.5	8.4	0.99
4.65	"	6.8	6.2	0.92
4.95	"	2.2	2.6	1.18
5.26	"	−1.1	−0.8	0.73
5.49	"	−2.9	−2.9	1.00
5.55	"	−4.6	−3.7	0.81
5.76	Phosphate	−5.9	−5.3	0.90
6.18	"	−8.5	−8.2	0.96
6.46	"	−9.8	−9.6	0.98
6.66	Phosphate-borate	−11.8	−10.8	0.92
7.00	Phosphate	−14.0	−12.8	0.91
7.47	"	−15.6	−14.9	0.95
8.27	Phosphate-borate	−18.0	−17.8	0.99
8.92	"	−19.7	−20.0	1.02
Mean				0.98

the dissociation curve. The ratio of mobility to net charge shows a satisfactory constancy from pH 4 to 9. The mean value of h/u over this range is 9.8×10^4.

A theoretical value of h/u may be computed on the basis of the Debye-Hückel-Henry theory of electrophoretic mobility (20). For a spherical ion, we have

$$u = \frac{he}{6\pi\eta r} \cdot \frac{\varphi(\kappa a)}{1 + \kappa a}$$

where e is the charge on the electron, η the viscosity, and $\varphi(\kappa a)$ is a function of κa whose value, at $\mu = 0.02$, is practically unity (21). It follows that

$$\frac{h}{u} = \frac{6\pi\eta r}{e} \cdot (1 + \kappa a)$$

If we set $a = r = 29.1$ Å. and assign to the solution the viscosity of water, we obtain for h/u the value of 8.2×10^4. This is about 85 per cent of that found experimentally. The discrepancy is not materially reduced by substituting a plausible value for the viscosity of the solutions whose mobilities were determined, nor by increasing a by an amount such as might reasonably correspond with the mean radius of the ions of the buffer solutions. It is significant that the theory approaches the experimental results more closely in the case of β-lactoglobulin than in that of egg albumin. For the latter protein, Longsworth (20) found that the theoretical value of h/u was only about 60 per cent of the observed value. The analysis of the situation in egg albumin was, however, complicated by the well known discrepancy between the isoelectric and the isoionic points. In the case of β-lactoglobulin this difficulty is not encountered.

SUMMARY

1. Studies are reported of the effects, on the dissociation curve of β-lactoglobulin, of temperature, the concentration of KCl, the concentration of protein, and the addition of formaldehyde.

2. The results are consistent with the presence of 58 carboxyl, 34 amino, 6 imidazole, and 6 guanidino groups in 1 mole (40,000 gm.) of β-lactoglobulin.

3. Analyses are reported of the amounts of histidine, arginine, lysine, total dicarboxylic acids, amino nitrogen, and amide nitrogen in β-lactoglobulin.

4. The shape of the dissociation curve and the effect of [KCl] thereon are described in terms of electrostatic interaction theory.

5. The ratio of net charge to electrophoretic mobility is substantially constant from pH 4 to 9. The value of this ratio is about 15 per cent greater than that computed from the Debye-Hückel-Henry theory of the mobility of spherical ions.

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THE ANTIGENIC STRUCTURE OF HEMOLYTIC STREPTOCOCCI OF LANCEFIELD GROUP A

X. THE PURIFICATION AND CERTAIN PROPERTIES OF THE GROUP-SPECIFIC POLYSACCHARIDE*

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In 1928 Lancefield (1) described a species-specific substance in acid extracts of hemolytic streptococci. No little evidence indicated the active substance to be a carbohydrate. The purest preparation, however, contained 4.2 per cent N and only 28 per cent reducing sugar (calculated as glucose). The yield was stated to be very small. In 1933 Lancefield showed (2) that this carbohydrate was contained only in hemolytic streptococci of human origin, and a serological method was described for grouping these and other hemolytic streptococci on the basis of similar specific substances. Five groups were recognized; most strains of human origin fell into one group which was designated Group A.

Recently Fuller (3) described another method, utilizing hot formamide to dissolve the streptococci, for obtaining these polysaccharides. The method is suitable for the small amounts of polysaccharide needed for serological grouping methods and appears to be particularly suitable for large scale extractions. The yields of polysaccharide obtained by Fuller have exceeded 1 per cent of the weight of dried streptococci used. Kendall, Heidelberger, and Dawson (4) in comparing the properties of the capsular and the somatic group-specific polysaccharide of Group A streptococci reported that the latter gave no test for uronic acid and contained P. The presence of an amino sugar has been reported (5).

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Fuller's method (3) was chosen for the isolation of the crude polysaccharide in the present study. Large amounts of streptococci were assured when it was found that the polysaccharide could be obtained from the organisms from which the type-specific substance had been extracted with dilute acid (6); the amount of the polysaccharide isolated was the same, on the dry weight basis, as from unextracted organisms. Purification was performed by chemical means and electrophoretic methods were used to follow the course of the purification.

EXPERIMENTAL

Preparation of Crude Polysaccharide—The procedure described by Fuller (3) was used, with some modifications. The acid-extracted streptococci (6) which serve as the starting material are dried with petroleum ether and extracted with formamide (1 gm. per 2.5 ml.) in a bath at 150° for 20 minutes. The precipitate which forms when 2.5 volumes of 95 per cent ethyl alcohol, containing 1 per cent of concentrated HCl, are added to the formamide extracts is discarded. The polysaccharide is precipitated with an equal volume of acetone. The precipitate is extracted with water, reprecipitated with 5 volumes of acetone containing 1 per cent of concentrated HCl, and dried with 95 per cent alcohol and ether. The preparations obtained by this procedure contain 2.1 to 6.5 per cent N; the three preparations analyzed contained 0.5 per cent P. These preparations are not completely soluble in neutral solvents. The insoluble material represents impurities.

A study of the conditions that would give preparations containing the least N has shown that the temperature of the bath should not exceed 150°. Further, the extent to which the extracts are clarified by centrifugation is important. When a slight turbidity remains, the maximum precipitation of impurities is obtained by the subsequent addition of alcohol. When these precautions are taken, the crude preparations usually contain 2.5 to 3.0 per cent N.

Properties of Crude Polysaccharide—The polysaccharide reacted with Group A streptococcal antisera in a final dilution exceeding $1:10^6$ and showed the marked prozone previously noted by others (1, 5). It gave no reaction with Groups B, C, D, E, F,

and H streptococcal antisera[1] or with antisera to *Streptococcus viridans* and smooth pneumococcus.

The dialyzability of the polysaccharide was determined by placing solutions in cellophane tubes and dialyzing 48 hours in the refrigerator against a relatively large volume of water. The dialysate was tested for the polysaccharide with a specific antiserum. The polysaccharide in the dialyzed solution was reprecipitated and the N and P content redetermined. In a typical experiment less than 1 per cent of polysaccharide appeared in the dialysate and the N content of the polysaccharide was reduced from 2.6 to 2.3 per cent; the P content remained at 0.50 per cent. The relative non-dialyzability of this polysaccharide was useful in further studies, since diffusible impurities could be eliminated by dialysis, and electrophoretic studies, where dialysis is a necessary step, could be made.

Electrophoretic Studies[2] *with Crude Polysaccharide*—From an observation made in electrophoretic studies of other streptococcal fractions (8, 9) it appeared that the group-specific polysaccharide was immobile in an electric field. Accordingly the use of electrophoresis appeared to be promising for obtaining a pure product for which chemical data could be obtained; this in turn would be useful in devising a method for chemical purification of more general applicability. It will be seen that electrophoretic purification was not successful, but that these studies did give us information as to the amount and nature of the impurities, which was useful in following the chemical purification.

The first electrophoretic experiment was performed with a 1.8 per cent solution of a preparation containing 2.1 per cent N, and 0.4 per cent P. The solvent[3] was phosphate buffer, pH 7.0, μ 0.02. The potential gradient was 6.2 volts per cm. The observations

[1] Streptococcal antisera of Groups A, B, C, D, E, F, and H were furnished through the generosity of the Lederle Laboratories, Inc.

[2] These studies were made possible through the generous cooperation of Dr. Florence B. Seibert of the Henry Phipps Institute and were performed with the Tiselius apparatus (7) of that institution.

[3] In this and subsequent experiments the solution was dialyzed against 2 liters of the solvent buffer for 24 hours. The conductivities of the polysaccharide solution and the buffer were determined after dialysis in several cases and found to be identical.

made with this preparation by the Svensson optical method (10) are shown in Fig. 1. The principal peak, the polysaccharide, was immobile. The small peak appearing on the ascending side which was eventually resolved into four peaks was shown, after separation and analysis of the solutions in the various compartments of the electrophoresis cell, to represent a relatively N-rich contaminant.

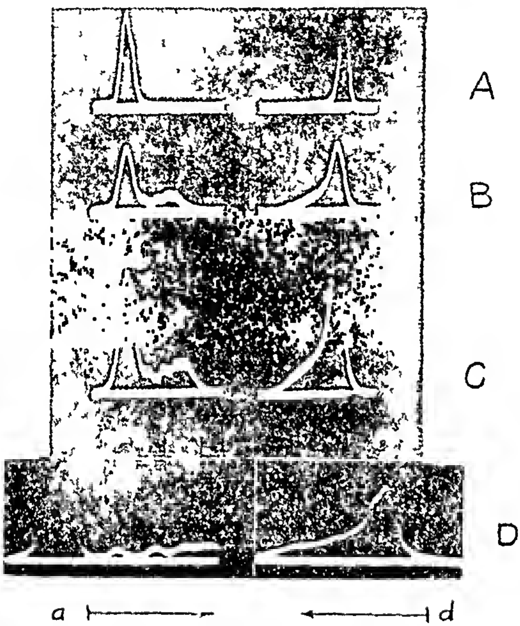

FIG. 1. Electrophoresis of the crude polysaccharide at pH 7.0. The time interval between *A*, when the electric current was started, and *B* was 41 minutes; between *B* and *C*, 8 minutes; between *C* and *D*, 78 minutes. For *D*, the mid-line was shifted and separate observations were made of the ascending and descending boundaries.

This experiment showed that purification by electrophoresis would be difficult or impossible because of poor resolution on the descending side. Even after 5 hours of electrophoresis the results were not very different from those shown in Fig. 1, *D*. The most rapidly moving peak on the ascending side had a mobility of -5×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} and the slowest of the mobile components had a mobility of -2×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}.

This experiment was repeated with a 1.0 per cent solution of polysaccharide containing 1.9 per cent N with similar results.

The same material was studied in acetate buffer, pH 4.5, μ 0.02. The principal component was immobile. Two negatively charged components were observed, the faster having a mobility of -2×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}, and one positively charged component. The mobilities and behavior on the descending side were such that electrophoretic separation of polysaccharide and contamination could not be performed.

Another experiment was performed with the polysaccharide in borate buffer, pH 9.0, μ 0.004. The concentration was 1.5 per cent. The results were very similar to those obtained at pH 7.0. The peaks appearing on the ascending side moved more rapidly, the fastest having a mobility of -10×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}, but no clear separation was obtained on the descending side. On the ascending side the polysaccharide had a slight movement toward the negative pole (downward), whereas on the descending side it had considerable movement toward the positive pole. In this last case the movement appeared to be due to the drag of the contaminant moving through the polysaccharide. This made prolonged electrophoresis impossible, since the boundaries were approaching each other.

Longsworth, Cannan, and MacInnes (11) have described apparent interactions of the proteins of egg white. The ascending and descending curves obtained in electrophoresis were far from being mirror images of each other. These interactions occurred between proteins of opposite charge and it cannot be said whether the interaction we have observed between a neutral polysaccharide and protein is similar. The interaction that they observed is favored by low ionic strength. More to the point are the recent studies of Seibert and Watson (12) in which interactions were observed between polysaccharide and nucleic acid and protein. The electrophoretic diagrams obtained for one fraction (S6) are almost identical with the diagrams obtained in our studies. Interaction was particularly striking with another fraction (S4) in which electrophoresis repeated five times, for a total of 114 hours, did not successfully separate the nitrogenous contamination from the polysaccharide. In their studies the resolution of the components of Fraction S4 was much poorer at an ionic strength of 0.1 than at 0.02. Our experiments were limited to buffers of ionic strength 0.02 and 0.004 (one experiment).

Chemical Purification of Polysaccharide—Phosphotungstic acid, lead acetate, and metaphosphoric acid have been used with little or no success in the attempt to remove the nitrogenous contamination revealed by electrophoretic experiments. Treatment with chloroform (13) effected a partial purification; polysaccharide preparations were obtained in which the N content was reduced to 1.9 per cent. Meyer *et al.* (14) had found such treatment not very successful when the nitrogenous contamination was of low molecular weight, particularly with neutral polysaccharides which seem to exert a protective action. The hot formamide method of preparation might be expected to give nitrogenous contamination of low molecular weight.

We have found Lloyd's reagent most effective in purifying this polysaccharide. The efficiency of this reagent in the purification of neutral polysaccharides has been pointed out by Meyer *et al.* (14). By its use white, granular preparations containing 1.72 per cent N and 0.70 per cent P have been prepared.

The most highly purified preparations are obtained as follows: The crude polysaccharide, previously described, is dissolved in water. The solutions are acid (blue to Congo red paper), and usually solution is complete except with the preparations containing 5 to 6 per cent N; any insoluble material is discarded. The solution is neutralized carefully with N HCl and the flocculent precipitates which form successively are discarded. No precipitate has been obtained above neutrality and this pH need not be exceeded. These precipitates have the N content of proteins and by their removal polysaccharide preparations are obtained containing 2.5 per cent N when precipitated with 5 volumes of acetone containing sufficient concentrated HCl to produce flocculation. These preparations are light buff-colored powders. Further purification is effected with Lloyd's reagent. An approximately 5 per cent solution of polysaccharide containing 5 per cent of glacial acetic acid is treated successively with 0.02 gm. of Lloyd's reagent per ml. of solution. The suspensions are shaken for several minutes. Successive treatments are performed by centrifuging and mixing the supernatant fluid with another portion of the reagent. This is done three to six times. Little or none of the polysaccharide is adsorbed on the reagent. The polysaccharide left in the fluid adhering to the reagent is washed off and recovered sep-

arately. The polysaccharide solution after treatment with Lloyd's reagent is dialyzed 16 hours with two successive 2 liter portions of distilled water. Very little polysaccharide is lost by this step and the content of inorganic material is reduced. The polysaccharide is precipitated with 5 volumes of acetone and sufficient concentrated HCl (4 to 6 drops to a volume of approximately 100 ml.) to obtain a flocculent precipitate. The dense, white precipitate when centrifuged coalesces and forms a viscous, clear, almost invisible film. The acetone is drained off thoroughly and the

TABLE I

Properties of Purified Polysaccharide

Specific reaction with Group A streptococcal antiserum	Reactive in final dilution exceeding 1:2,000,000
Relative viscosity of solutions in 0.90% NaCl at 25°	0.1%, 1.01; 0.5%, 1.04; 1.0%, 1.09
Optical rotation at 24°; 2.0% solution in 2 dm. tube; $\alpha_D = -2.87°$	$[\alpha]_D^{24} = -71.5°$
Reducing value after hydrolysis, as glucose, by Hagedorn-Jensen method*	87.2%
Electrophoretic mobility, μ 0.02, pH 7.0, phosphate	Negligible mobility
Electrophoretic mobility, μ 0.02, pH 9.0, borate	Two principal components: (1) negligible mobility; (2) -7.0×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}
Nitrogen content	1.72%
Phosphorus content	0.70%
Orcinol reaction (pentose), 5 mg. sample	Positive†
Ehrlich's diazo reaction (diazobenzenesulfonic acid), 5 mg. sample	Negative
Test for amino sugar (*p*-dimethylaminobenzaldehyde) (15)	Before hydrolysis, negative; after hydrolysis, positive‡

* Sample hydrolyzed in closed tube with 1.0 ml. of concentrated HCl and 5.0 ml. of H_2O for 2 hours at 100°.

† The green color appeared much more slowly than with the same weight of arabinose and did not undergo a final change to blue as did arabinose.

‡ The hydrolyzed solutions prepared for the determination of the reducing value were used.

polysaccharide dried with absolute ethyl alcohol. The viscous film becomes brittle and easily powdered in absolute alcohol. The pure white, granular product is dried in a vacuum desiccator over P_2O_5 and ascarite. The properties of the polysaccharide prepared by this means are given in Table I. This material was subjected to electrophoresis in 1.0 per cent concentration in borate buffer, pH 9.0, and phosphate buffer, pH 7.0; the ionic strength in both

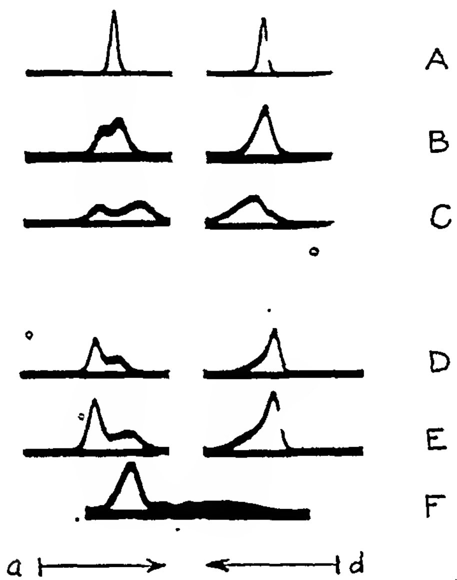

FIG. 2. Electrophoresis of the purified polysaccharide. The *A*, *B*, and *C* curves were obtained in borate buffer at pH 9.0; the *D*, *E*, and *F* curves in phosphate buffer at pH 7.0. The times elapsed between *A* and *B* and between *B* and *C* were 24 and 22 minutes, respectively. *D* was obtained 20 minutes after electrophoresis had begun; the times elapsed between *D* and *E* and between *E* and *F* were 10 and 52 minutes, respectively.

cases was 0.02. The results are shown in Fig. 2. The significance of the new negatively charged peak which appeared in the borate buffer[4] on the ascending side is considered in the discussion. The transformation of the polysaccharide which occurs in borate buffer, pH 9.0, μ 0.02, is largely reversed in a phosphate buffer at pH 7.0.

[4] The previous experiment with borate buffer at this pH was performed in more dilute buffer (μ 0.004), which may account for the non-appearance of this component in the same striking amount.

The material recovered from the experiment at pH 9.0 was subjected to electrophoresis in phosphate buffer, pH 7.0, μ 0.02, and the previously predominant mobile peak was not seen. The picture obtained after 81 minutes of electrophoresis was very similar to that shown in Fig. 2, *F*, with perhaps more of the most mobile component showing.

DISCUSSION

The electrophoretic data are of interest in relation to the purity of the final polysaccharide preparation. The appearance of large amounts of a new component in the borate buffer[5] at pH 9.0 raises the question whether some of this may be present at other pH values and other conditions. It may represent complex formation with the borate. However, it is of interest that extrapolation of the mobilities of the most mobile component in acetate at pH 4.5 and in phosphate at pH 7.0 leads to a mobility identical to that measured at pH 9.0 in borate. The fastest component in the experiments at the lower pH values may represent transformed polysaccharide also. In any case some contamination is present, since the less mobile peaks clearly seen in *D* of Fig. 1 are visible also, although to a smaller extent, in *F* of Fig. 2. The isoelectric point of the fastest component is quite acid, pH 2.5, based on a linear extrapolation of the mobilities at pH 4.5, 7.0, and 9.0.

The amino sugar test (see Table I) was negative with the unhydrolyzed polysaccharide but after hydrolysis a strongly positive test was obtained; 1.0 mg. of hydrolyzed polysaccharide gave about the same amount of cherry-red color as 0.2 mg. of glucosamine. Although we previously (17) expected to obtain the polysaccharide free of N, it now appears, from the earlier discussion of the electrophoretic data and the strongly positive amino sugar test, that most of the 1.72 per cent N of the purified preparations can be regarded as part of the molecule.

The P also seems to be a part of the polysaccharide molecule. The basis for this conclusion is as follows: The amount of P present, if as contaminating nucleic acid, would represent 7.0 per

[5] Watson (16) has made somewhat similar observations with a tuberculin polysaccharide. At pH 9.3 in borate buffer the polysaccharide separated into two definite components, whereas at the other pH values there was simply a heterogeneous curve.

cent of nucleic acid which should have been detected in electrophoresis with the sensitive optical system used for observation. Furthermore, when subjected to electrophoresis, the mobility of the nucleic acid being high (8), a diminution of the P content would be expected. However, the material recovered after electrophoresis contained the same amount of P as the starting material. The same result was obtained in both the borate and phosphate buffers; hence it does not seem likely that the P represents absorbed phosphate, since if it did it would likely increase when in contact with the phosphate buffer. Kendall *et al.* (4) have stated that this polysaccharide contains P; the evidence for their conclusion was not given. The P in biological compounds has always been found as phosphoric acid and it is unlikely that the primary phosphoric acid group is esterified to give a neutral polysaccharide. It may be that an inner salt (zwitter ion) is formed, which would account for the observed neutrality of this polysaccharide.

The relative viscosities of solutions of this polysaccharide are lower than any obtained by Brown (18) in her extensive studies of the pneumococcal type-specific polysaccharides.

The unusually high levorotation is striking and should be useful in following the purification of this polysaccharide. The preparations of Lancefield (1), isolated from the streptococci by a different procedure, were levorotatory also and had a specific rotation of 33°.

SUMMARY

The streptococcal Group A-specific polysaccharide was isolated from acid-extracted organisms by the formamide method of Fuller. After purification by fractionation at varying pH and the use of Lloyd's reagent, a product was obtained which was reactive in dilutions exceeding 1:2,000,000 with specific antisera and contained 1.72 per cent N, 0.70 per cent P, and a reducing value (as glucose) of 87.2 per cent. 1 mg. of the hydrolyzed polysaccharide gave about the same amount of color in an amino sugar test as 0.2 mg. of glucosamine. The specific rotation was −71.5°. It was immobile in an electric field. The diffusion through cellophane casing was slight and the relative viscosity was very low.

Addendum—Physicochemical measurements have been made with this polysaccharide in the Department of Chemistry of the University of Wis-

consin by Miss Ellen Burtner under the direction of Professor J. W. Williams. Diffusion measurements have shown that the polysaccharide is fairly homogeneous and in the ultracentrifuge it appeared to be a single substance. The diffusion constant (D_{20}) is 10.5×10^{-7} cm.^{2} per second and the sedimentation constant (s_{20}) is 1.35×10^{-13} cm. per second per dyne. Assuming the partial specific volume to be 0.62 (such a value was obtained for a tuberculin polysaccharide (Seibert *et al.* (19)) which in other physical properties appears to be very similar. These polysaccharides are, however, immunologically distinct.) the molecular weight is about 8000 and the Svedberg dissymmetry number, f/f_0, is 1.6. The large value for f/f_0 indicates that the molecule deviates from a spherical shape or that it is hydrated or both. If the value for f/f_0 is due to the former, the ratio of the major to minor axis would be about 11.0:1. However, the polysaccharide is probably hydrated to some extent in view of the tenacity with which it holds water. These data will be published in detail elsewhere as part of the program of study of bacterial polysaccharides in progress in the University of Wisconsin laboratory. The interest of Professor Janet R. McCarter of the University of Wisconsin in these studies is gratefully acknowledged.

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MICRODETERMINATION OF TRIPHOSPHOPYRIDINE NUCLEOTIDE

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In the studies which led to the discovery and isolation of triphosphopyridine nucleotide (TPN), Warburg, Christian, and Griese (1) determined the TPN quantitatively by a method which involved its oxidation by the old yellow enzyme and oxygen. This manometric test involves relatively large concentrations of the reacting constituents and the old yellow enzyme is not specific for TPN; it has also been used (2) in a quantitative test for diphosphopyridine nucleotide (DPN). Cytochrome *c* reductase, on the other hand, is specific toward TPN; it is inactive with the coenzyme DPN. The system, cytochrome *c*-cytochrome *c* reductase-TPN-*Zwischenferment*-glucose-6-phosphate, which was used in the test for cytochrome *c* reductase, can also be employed as a test for any one of the constituents of the system.

In this paper we are giving the conditions necessary for the employment of this system for the quantitative determination of TPN. The over-all reaction is the reduction of cytochrome *c*, observed spectrophotometrically, by glucose-6-phosphate; the rate at which the cytochrome *c* is reduced is proportional to the TPN concentration.

The test as described here is very sensitive; 0.025 γ of TPN can be determined with an error of less than 10 per cent. It should therefore be of use in biological or clinical studies in which only very limited amounts of material are available.

Test Substances—The components of the test were prepared as previously described (3). In order to simplify the preparation of the test substances, partially purified samples of *Zwischenferment* and of the cytochrome reductase may be used. For this study,

Analytical Test

Wave-length, 550 mμ; temperature, 25°; 1.0 ml. of 0.025 M phosphate buffer, pH 8.3; 0.75 mg. of glucose-6-phosphate; 2.5 mg. of *Zwischenferment*; 1.2 mg. of cytochrome *c*; 0.25 mg. of cytochrome reductase; 5 $\times$ 10^{-5} mg. of triphosphopyridine nucleotide.

$$\text{CyFe}^{+++} = \frac{1/l \times \log I_0/I - \alpha_{\text{reduced}} \times C}{\alpha_{\text{oxidized}} - \alpha_{\text{reduced}}} = 10.86 \times 10^{-8} - 16.83 \times 10^{-8} \times \log \frac{I_0}{I} \left[\frac{\text{moles}}{\text{ml.}}\right].$$

C = total cytochrome concentration = 7.17 $\times$ 10^{-8} (mole $\times$ ml.$^{-1}$).

CyFe^{+++} = concentration of ferricytochrome *c* (mole $\times$ ml.$^{-1}$).

l = length of absorption cell = 0.32 cm.

α_{reduced} (4) = absorption coefficient of ferrocytochrome *c* = 0.281 $\times$ 10^8 (cm.^{2} $\times$ mole^{-1}).

α_{oxidized} = absorption coefficient of ferricytochrome *c* = 0.0956 $\times$ 10^8 (cm.^{2} $\times$ mole^{-1}).

Time	$\frac{I_0}{I}$	Log $\frac{I_0}{I}$	CyFe^{+++}	Log CyFe^{+++} + 8	Δ log CyFe^{+++}	$\frac{\Delta \log \text{CyFe}^{+++}}{\Delta t}$
min.			*mole $\times$ ml.$^{-1}$*			*min.$^{-1}$*
0	1.655	0.219	7.17 $\times$ 10^{-8}	0.855		
1	1.715	0.234	6.92	0.840	0.015	0.015
2	1.770	0.248	6.68	0.825	0.030	0.015
3	1.825	0.261	6.46	0.810	0.045	0.015
4	1.875	0.273	6.27	0.797	0.058	0.015
5	1.920	0.283	6.10	0.785	0.070	0.014
6	1.985	0.298	5.85	0.767	0.088	0.015

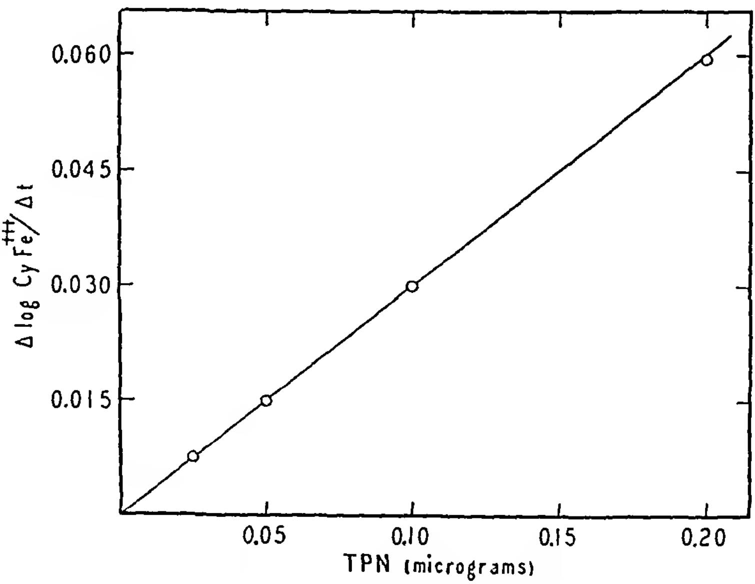

FIG. 1. The rate of reduction of ferricytochrome *c* as a function of the triphosphopyridine nucleotide concentration.

60 mg. of the cytochrome reductase preparation, obtained from Step 4 in the previously described method of preparation (3), were dissolved in 3.5 ml. of water by slowly adding 0.50 ml. of 0.1 N potassium hydroxide. The solution was dialyzed in a cellophane tube against 3×10^{-3} N ammonium hydroxide, for 24 hours, at 0°, to remove traces of TPN. For each experiment 0.04 ml. of the dialyzed solution, equivalent to 0.25 mg. of protein, was used.

In Fig. 1 the rate of reduction of cytochrome *c*, $\Delta \log \mathrm{CyFe}^{+++}/\Delta t$, is plotted, when different amounts of TPN are added to the test solution. The proposed method allows the determination of about 100 times smaller amounts of TPN than the test which includes the old yellow enzyme and oxygen. This is partly due to the higher activity of the cytochrome reductase and partly to the higher sensitivity of the optical method. With as much as 0.40 γ of diphosphopyridine nucleotide, no reduction of cytochrome could be observed.

SUMMARY

A spectrophotometric test, which allows the accurate determination of 2×10^{-2} to 20×10^{-2} γ of triphosphopyridine nucleotide in the course of a few minutes, has been described. *Di*phosphopyridine nucleotide is inactive in this test.

We wish to acknowledge our indebtedness to the Rockefeller Foundation for a grant-in-aid which made this work possible and to the Works Progress Administration for help during the course of this investigation.

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ON THE ESTIMATION OF ALLANTOIN BY THE RIMINI-SCHRYVER REACTION*

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The need for a rapid, accurate, and sensitive method of estimating allantoin is apparent from the numerous procedures which have been proposed within the past decade. The method should be applicable to small amounts of urine or blood in the routine analysis of metabolic experiments. This has become apparent to us in the course of investigating the purine metabolism of the Dalmatian coach hound (Young, Conway, and Crandall, 1938).

Dissatisfaction with the accuracy and elegance of three modern methods with which we have had experience, *viz.* those of Christman (1926), Larson (1931–32), and Mourot (1935), has led us to investigate the conditions necessary to satisfy the above requirements. All procedures which require the removal of interfering substances from the urine before the final estimation, as in the original Wiechowski procedure and in both the Larson and Christman methods, are objectionable because of bulky precipitates with the resulting danger of adsorption or occlusion of allantoin. Although the Larson method is a rapid one, we have found that the values obtained are generally higher than by the procedure proposed. This is presumably due to the presence in urine of reducing compounds which are not removed prior to the color reaction. The Christman method on the other hand is a very tedious one, not well suited to metabolic experimentation in our experience. It tends to give values which are too low in our hands.

The sensitivity of the Rimini reaction for formaldehyde as

* A preliminary report was made to the Sixteenth International Physiological Congress at Zurich (*Kongressbericht II*, 126 (1938)).

modified by Schryver (1910) is 1 in a million. This reaction is directly applicable to glyoxylic acid, as shown by Fosse and Hieulle (1924), and has been made the basis of quantitative methods for the estimation of allantoin in urine (Fosse *et al.*, 1930, 1931; Ro, 1931; Mourot, 1935; Borsook, 1935; Paget and Berger, 1938). In this procedure allantoin is converted to allantoic acid by the allantoinase of soy bean. The allantoic acid is hydrolyzed to urea and glyoxylic acid by dilute hydrochloric acid, the phenylhydrazone is formed, and a color developed by oxidation with ferricyanide. The color is measured against a standard of allantoic acid, spectrophotometrically by Fosse *et al.* (1931) and Borsook (1935), and in a Duboscq colorimeter by Mourot (1935) and Ro (1931).

By a systematic investigation of some of the conditions controlling these reactions it has been possible to simplify the procedure considerably and to adapt the method to the ordinary colorimeter with an accuracy of ±5 per cent.

EXPERIMENTAL

The modification of Mourot (1935) of the Fosse *et al.* (1931) procedure has been tested by us on pure solutions of allantoin containing 10 to 50 mg. per 100 ml. The amounts estimated represented recoveries of 56 to 94 per cent, with an average of 78 for fifteen determinations. Duplication of results was difficult and there were differences in shade of the color developed from brownish to vivid crimson. Accepting this technique, however, as a guide, we have investigated the various steps in the procedure.

The chemicals of doubtful purity, *viz.* phenylhydrazine hydrochloride, potassium ferricyanide, and potassium allantoate, were first recrystallized several times to be certain that impurities in these chemicals were not responsible for the discrepancies. Fresh solutions were prepared each day.

As a result of the investigation of these variables in the procedures published by Fosse and his colleagues we have devised the following method of estimating allantoin, using a Klett colorimeter. The reasons for our modifications will be apparent from the experimental details given later.

Method

Reagents—

1. Sodium hydroxide, 0.5 N.
2. Hydrochloric acid, 0.5 N and concentrated (sp. gr., 1.19).
3. Phenylhydrazine hydrochloride, 0.33 per cent. 0.05 gm. in 15 ml. of water, prepared daily.
4. Potassium ferricyanide, 1.67 per cent. 0.25 gm. in 15 ml. of water, prepared daily.
5. Potassium allantoate standard, 20.0 mg. in 1000 ml. of 0.01 N sodium hydroxide, prepared every 3 months.

Procedure—An aliquot of urine containing 0.05 to 1 mg. of allantoin (usually 1 to 2 ml.) is transferred to a 50 ml. volumetric flask and diluted to volume with distilled water. 5 ml. of the diluted urine are pipetted into a Pyrex tube graduated at 25 ml., such as a blood urea tube, 20 × 150 mm., 1 ml. of 0.5 N NaOH is added, and the tube placed in a vigorously boiling water bath for 7 minutes. On removal it is immersed in a water bath at 20°.

1 ml. of 0.5 N HCl is added with 5 drops additional to adjust acidity to approximately 0.02 N. 5 ml. of the standard solution of allantoate are pipetted into a separate tube and 5 drops of 0.5 N HCl added. Then 1 ml. of the phenylhydrazine solution is added to each tube. The tubes are shaken and placed in a boiling water bath for *exactly* 2 minutes. They are then *immediately* plunged into an ice-salt bath at approximately −10° and chilled to incipient freezing in about 3 minutes.

On removal from the bath, 3 ml. of concentrated hydrochloric acid, also chilled to −10°, are added to each tube and 1 ml. of potassium ferricyanide solution. The contents are well mixed. After 30 minutes the tubes are filled to the mark with distilled water and compared in a Duboscq colorimeter.

The calculation is therefore

$$\frac{S}{U} \times 0.1 \times 0.738 \times \frac{50}{5} \times \frac{100}{X}$$

or simply $S/U \times 73.8/X$ = mg. of allantoin in 100 ml. of urine, where S = the reading of the standard, U = the reading of the unknown, X = the number of ml. of urine used for initial dilution

to 50 ml., and 0.738 = the factor for the conversion of allantoate to allantoin.

The concentration of the standard may be varied between the limits of 5 and 100 mg., as required in ordinary colorimetric practice.

Preparation of Potassium Allantoate—As a standard, potassium allantoate is better than either allantoin or allantoic acid because of its stability in solution and ease of purification. We have prepared it as follows: 2.8 gm. of allantoin are stirred into a solution of 20 ml. of 1 N KOH at 75° and the solution is maintained at this temperature for 30 minutes. After cooling, 200 ml. of 95 per cent ethyl alcohol are added and the cloudy solution is placed in the ice box overnight, when crystallization takes place. The yield is the theoretical 3.45 gm. under these conditions. One recrystallization is desirable; a minimum quantity of warm water is used and alcohol added in the ratio of 20:1. The crystals are dried at 100° and powdered. M.p. 183–185° (uncorrected) with decomposition. This procedure is a modification of the method of Ponomarew (1878).

Conversion of Allantoin to Allantoic Acid—In the procedure adopted sodium hydroxide is used for this purpose. The use of the allantoinase as the powdered bean of *Soja hispida* in a medium of ammonium sesquicarbonate was introduced by Fosse and Brunel (1929), and applied by Fosse *et al.* (1930) and Mourot (1935) to the estimation of allantoin in urine. Ammonium sesquicarbonate is not listed in English or American chemical catalogues and there appears to be doubt as to the existence of such a substance. Under the original conditions pH 9.5 was obtained with a mixture of ammonium bicarbonate and carbonate to the formula $2NH_4HCO_3 \cdot (NH_4)_2CO_3$. Using pure solutions of allantoin, we have determined the effect of variation in pH with allantoinase at 40° for an interval of 12 hours. Table I records these results and demonstrates the importance of a pH of 12 to 13.

In brief the technique employed was as follows: Potassium cyanide (0.075 gm.) and ammonium sesquicarbonate (0.2 gm.) were added to a solution containing a minimum of 0.5 mg. of allantoin in 100 ml. Soy bean powder (1 gm.) and chloroform (10 drops) were added and the mixture incubated at 40° for 12

hours. The solution was then centrifuged and 1 N sulfuric acid added to an aliquot (5 to 10 ml.) until acid to litmus. Then followed the addition of minimal amounts of sodium tungstate (5 per cent) and $\frac{2}{3}$ N sulfuric acid to remove the soy bean protein. The material was filtered into a volumetric flask and the precipitate washed with 0.01 N hydrochloric acid. A volume was so formed as to provide a liter of allantoic acid of not more than 15 mg. per liter. 2 ml. of this solution were treated with 2 drops of phenylhydrazine hydrochloride (1 per cent) in a tube graduated at 20 ml., placed in a boiling water bath for 2 minutes, cooled in tap water, and 3 ml. of concentrated hydrochloric acid and

TABLE I

Influence of pH on Hydrolysis of Allantoin with Allantoinase

pH	Allantoin		Recovery
	Initial	Estimated	
	mg. per 100 ml.	*mg. per 100 ml.*	*per cent*
8.0	18.2	8.5	47
8.4	19.7	10.8	55
9.0	18.9	9.8	52
10.0	21.7	14.6	67
10.4	15.5	10.8	70
11.5	17.3	14.3	83
12.0	18.0	18.1	100
12.9	18.7	18.9	101

2 drops of potassium ferricyanide (5 per cent) were added. 2 ml. of standard potassium allantoate were treated similarly and colors compared after 30 minutes.

The high pH of the reaction suggested to us the importance of alkali in the estimation. The omission of the soy bean meal did not affect the reaction whatever at this pH. The allantoin can be completely converted to allantoate at pH 12 in about 6 minutes in a boiling water bath. Heating for longer periods up to 20 minutes does not affect the allantoate formed. The same result may be obtained by the use of lower temperatures by prolonging the time, as is shown in Table II. This mode of conversion of allantoin to allantoate has been used previously by Fosse and Bossuyt (1929) employing 0.1 N KOH at 60° for 2 hours, and by

Allen and Cerecedo (1931) who used 0.2 N KOH at 70° for 2 hours. Our experiments have convinced us that it is possible to shorten the procedure by raising the temperature without loss of accuracy. There is occasionally a disadvantage if much pigment is formed in the urinary sample.

Hydrolysis of Allantoic Acid—The reaction is usually carried out at 100° in the presence of dilute hydrochloric acid, 0.01 to 0.1 N, and phenylhydrazine hydrochloride. Experiments were tried with pure solutions of allantoate heated at 100° for different

TABLE II

Influence of Temperature on Hydrolysis of Allantoin with NaOH Only at pH 12

Temperature	Time	Allantoin		Recovery
		Initial	Estimated	
°C.	min.	mg.	mg.	per cent
70	15	29.6	21.3	76
70	120	30.0	29.4	98
80	10	29.5	23.9	81
80	15	23.2	22.7	97
80	30	23.2	22.4	96
80	60	24.0	24.6	102
90	10	29.8	27.1	90
90	15	32.6	31.5	97
90	30	26.2	27.1	103
100	5	10.0	9.5	95
100	8	10.0	9.8	98
100	12	10.0	9.8	98
100	20	10.0	9.7	97

intervals in various concentrations of acid and placed subsequently in a bath at 8°. The standard tube was heated for 2 minutes only, as a longer interval caused a decrease in the intensity of the color. A concentration of 0.04 to 0.05 N HCl was most suitable. By separating the addition of acid and phenylhydrazine it was possible to show that the hydrolysis was completed in 1 to 2 minutes at 100° and that the products were not affected by 10 minutes at this temperature. The hydrazine reaction, however, was appreciably disturbed by longer heating than 3 minutes at 100°, as may be seen in Table III. It may, however, be completed at a lower temperature after the hydrolysis

of the allantoic acid. This is important in the event of the presence of compounds such as sugars which react with phenylhydrazine.

The apparent instability of the reaction between glyoxylic acid and phenylhydrazine hydrochloride suggested a trial of lowering the temperature of the solution promptly to inhibit side reactions.

The effect of rapidity of cooling was therefore next investigated following the formation presumably of the glyoxylic phenylhydrazone. Color intensities were referred to a standard cooled for 3 minutes in an ice-salt bath at −10°. All tubes were equilibrated in a water bath at 20° as soon as removed from the cooling medium. Alcohol and solid carbon dioxide mixtures provided baths ranging from −60° to −20°.

Table III

Hydrolysis of Allantoic Acid and Reaction of Glyoxylic Acid Formed with Phenylhydrazine

Temperature	Time	Allantoate estimated (initial 38 0 mg)	Recovery
°C.	min.	mg	per cent
100	1	36.6	96
100	2	38.0	100
100	3	36.6	96
100	5	34.8	91
90	2	38.8	102
90	20	0	0
50	5	37.2	98
40	5	38.0	100
30	5	36.8	97
25	15	36.8	97
20	20	37.2	98

Rapid cooling increased the intensity of the final color. Prolonged cooling in the freezing mixtures beyond 3 minutes was without effect. More elevated temperatures than −10° tended to cause brownish colorations. Lower temperatures increased the color intensity so that at −60° it was 15 per cent higher than at −10°. The *rapidity* of cooling is therefore an important factor in technique, as has been previously noted only by Borsook (1935).

Stability of Standard Solutions of Allantoate—By the procedure

described above solutions of potassium allantoate prepared on the same day have agreed well in titer. If, however, such solutions were allowed to age at a neutral reaction and were compared with fresh standards, a progressive decline in titer was observed. In a solution of 1 N HCl this decline was very rapid. In acidities of 0.1 to 0.01 N HCl there was an increase in titer of approximately 10 per cent during the first 24 to 48 hours followed by a decline to within 4 per cent of the original value and this value remained constant for a period of 2 months. In an alkalinity of 0.01 N NaOH the titer has been observed to remain constant for a period of at least 3 months. This is the more logical medium for the standard allantoate, rather than acid with its tendency to induce hydrolysis to glyoxylic acid and urea with subsequent decomposition of the glyoxylic acid.

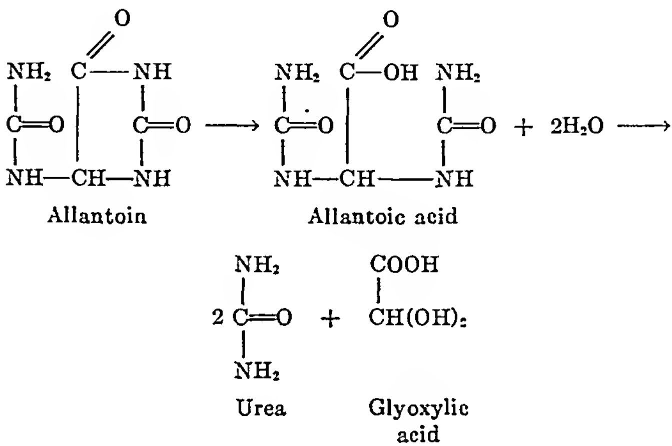
Color Intensity and Concentration of Allantoate—To determine the limits of concentration of allantoate and their relation to the intensity of the color developed, standard solutions containing 5 to 100 mg. of potassium allantoate per liter were prepared. 5 ml. samples were used to develop the color in the usual way and they were compared with a suitable standard set at 20 mm. according to the usual colorimetric practice. Below 5 mg. the color was rather weak for comparison and above 100 mg. pigmented precipitates formed. Thus the limiting concentrations for a single determination by this procedure would be 0.025 to 0.5 mg. of potassium allantoate or approximately 0.02 to 0.4 mg. of allantoin.

Stability of Color—By comparison with a standard prepared every 30 minutes it was possible to determine the rate of fading of the color with time. The maximum color is developed in about 20 minutes and in 60 minutes there is a loss of 8 per cent, in 90 minutes 15 per cent, in 120 minutes 22 per cent.

Specificity of Rimini-Schryver Reaction—The specificity of this reaction and its chemistry have been little investigated. The original Rimini reaction was devised for the detection of formaldehyde by the use of phenylhydrazine hydrochloride, sodium nitroprusside, and sodium hydroxide. Schryver (1910) modified the test and increased its sensitivity greatly by using phenylhydrazine and potassium ferricyanide in a medium of concentrated hydrochloric acid. He offered the explanation without evidence that "the reaction is due to the formation of a condensation prod-

uct of formaldehyde and phenylhydrazine, which, on oxidation, yields a weak base; the latter, in presence of excess strong acids, yields salts, which readily undergo hydrolytic dissociation on dilution." Fosse and Hieulle (1924) showed this reaction to be given by glyoxylic acid in 1 part in 1 million.

The stoichiometrical basis for the reaction, however, depends primarily on the isolation of urea (Fosse and Bossuyt, 1927;



I

$$\begin{matrix} CHO \\ | \\ COOH \end{matrix} + C_6H_5NH \cdot NH_2 \longrightarrow \begin{matrix} CH{:}N \cdot NH \cdot C_6H_5 \\ | \\ COOH \end{matrix} + H_2O$$

$$+ K_3Fe(CN)_6 \longrightarrow \text{chromophore}$$

II

Fosse, Brunel, and de Graeve, 1929; Allen and Cerecedo, 1931) in conformity with equation (I). The quantitative character of the reaction between the glyoxylic acid formed and phenylhydrazine as in equation (II) rests on slender evidence.

In one experiment only Fosse and Hieulle (1924) demonstrated qualitatively that a specimen of calcium glyoxylate of unknown purity gave a positive color reaction. As we were interested in determining the conditions for the greatest sensitivity of the reaction, it was of importance to determine the quantitative character

of this reaction. To this end after many difficulties a specimen of glyoxylic acid was prepared by the electrolytic reduction of oxalic acid (Mohrschutz, 1926) and recrystallized several times. This material proved to be highly hygroscopic and was dried over phosphorus pentoxide in a weighing bottle. 1.04 mg. were weighed out on a micro balance and dissolved in 100 ml. of water. An aliquot of this solution was used as standard against the pure potassium allantoate standard with our technique. This was also carried out with a specimen of calcium glyoxylate prepared in the course of isolating the glyoxylic acid. On the assumption that 1 molecule of allantoate gives 1 molecule of glyoxylic acid and that the molecular weight of the latter is 74 as used, the results of the comparison agree reasonably well. 1 mg. of standard potassium allantoate should require 0.346 mg. of glyoxylic acid to provide the same depth of color. It actually required 0.361 mg., a difference of only 4.3 per cent. With calcium glyoxylate the calculated equivalent is 0.434 mg. and it required 0.495 mg. of our specimen, a difference of 14 per cent, due either to impurity in the specimen or side reactions.[1]

We have tested a large number of compounds which might, directly or indirectly, act as chromogens when the reaction was applied directly to urine. In general the test was made by the identical technique given above. In those cases in which pigment was formed it was matched when possible against a comparable standard and the molar ratio to allantoin calculated.

The following compounds gave no reaction: glucose, fructose, creatine, creatinine, hydantoin, guanidine, uracil, guanine, alanine, acetone, and hippuric, oxalic, diacetic, nicotinic, and citric acids.

Several compounds tended to form brownish colorations under the conditions given: xanthine, hypoxanthine, glycerol, pyruvic, malic, and tartartic acids, and aldehydes generally.

A few compounds produced a positive, pink coloration: alloxan, alloxantin, glycine, chloral, and lactic and uric acids. Of these the only important compound as a factor in disturbing the spec-

[1] We wish to acknowledge our indebtedness to Miss Helen Wentworth for the preparation of glyoxylic acid and the comparisons cited.

ificity of the method is uric acid. The stoichiometrical ratios with allantoin as standard were as follows: uric acid 1:8, alloxan 1:90, alloxantin 1:234, lactic acid 1:580, glycine 1:890.

Aldehydes gave some distinctive coloration when the initial treatment with alkali was omitted, as in the Schryver technique. The following were tried: formaldehyde, acetaldehyde, benzaldehyde, salicylaldehyde, furfuraldehyde, paraldehyde, and metaldehyde. The reaction proceeded better in a medium of ethyl alcohol. Only formaldehyde gave a pure pink, the others producing varying shades of orange to green.

As a check on the above lists a "synthetic" urine of pH 7 was prepared to simulate dog urine (Hawk and Bergeim, 1937). The percentage composition was as follows: urea 3.00, creatine 0.120, creatinine 0.120, oxalic acid 0.002, hippuric acid 0.070, uric acid 0.008, glucose 0.100, allantoin 0.140, NaCl 1.21, $KHSO_4$ 0.43, NH_4Cl 0.22, $CaH_4(PO_4)_2 \cdot H_2O$ 0.44, $MgSO_4$ 0.20, H_2O to 100 ml. This fluid in the presence of all constituents gave a titer of 142 mg. of allantoin, a result that is practically identical with that added. In the absence of uric acid it was 142 mg. In the absence of uric acid and glucose the value obtained was 137 mg.

Accuracy of Method—A series of determinations by our technique on pure solutions of allantoin varying in concentration from 1 to 68 mg. per 100 ml. is recorded in Table IV. The limits of error may be placed at approximately ±5 per cent. The recovery of pure allantoin added to specimens of dog urine, previously analyzed, is also shown in Table IV.

Comparison of Methods—There have been few comparisons of the various methods available for the estimation of allantoin in urine. Investigators proposing new techniques have frequently failed to publish comparisons of their results with those obtainable by other methods. Larson (1931–32) did compare his method with the Wiechowski-Handovsky procedure on twelve samples of rat urine with variations in agreement from −24.0 to +31.7 per cent. Bergami, Baer, and Boeri (1936) have compared the methods of Wiechowski-Handovsky, Larson, Fosse-Bossuyt, and Ro on rat urine. They draw the conclusion that the modification of Ro (1931) of the Fosse and Bossuyt (1929) procedure is to be preferred because of rapidity and accuracy. However, they do not

publish their quantitative findings by these methods, except in one case to show that the Larson method gave results that were very high relative to the Fosse procedure.

A comparison of the values obtainable by our technique and the methods of Larson (1931–32) and of Christman (1926) both before and after the addition of allantoin has therefore been carried out on urinary specimens from dogs, cats, and rabbits. The results are given in Table V.

TABLE IV

Recovery of Allantoin

Allantoin			Recovery	Remarks
Initial	Added	Estimated		
mg.	*mg.*	*mg.*	*per cent*	
1.0		0.94	94	Pure solution
2.0		1.90	95	" "
4.0		3.90	98	" "
6.0		5.72	95	" "
16.2		16.6	102	" "
27.0		27.6	102	" "
36.5		35.8	98	" "
48.2		47.7	99	" "
67.6		68.3	101	" "
41.4	10.2	51.8	102	Dog urine
62.6	12.9	75.8	102	" "
28.9	15.8	44.5	99	" "
72.3	20.8	94.0	104	" "

From these results it would seem justified to draw the following conclusions. (1) The Larson method gives values that are higher than those by the authors' procedure. This was found for all urines examined except for one in which the values were identical. The average result was 33 per cent higher for all specimens. In the two samples of cat urine it was, however, only 12 per cent higher. In seven samples of dog urine it was 22 per cent higher. The agreement was poorest for rabbit urine. (2) The Christman method gives values that are systematically lower than those by the authors' procedure. They varied from 4 to 55 per cent, averaging 35 per cent lower for all urines examined. There does not appear to be any particular species difference in

agreements. (3) In the recovery of added allantoin the Larson method and the authors' procedure agree reasonably well, especially on rabbit urines. The recovery by the authors' method

TABLE V

Comparison of Methods in Recovery of Allantoin from Urine

Experiment No.	Method	Allantoin			Recovery	
		Original	Added	Total		
		mg.	*mg.*	*mg.*	*mg.*	*per cent*
1. Dog	Authors	393	52.5	447	54	103
	Larson	530	52.5	587	57	109
	Christman	275	52.5	324	49	93
2. "	Authors	273	99.4	380	107	108
	Larson	270	99.4	406	136	137
	Christman	157	99.4	237	80	81
3. "	Authors	161	25.4	184	23	91
	Larson	263	25.4	296	33	130
	Christman	82	25.4	89	7	30
4. "	Authors	298	53.8	352	54	101
	Larson	348	53.8	392	44	81
	Christman	217	53.8	266	49	91
5. Cat	Authors	177	30.6	205	28	92
	Larson	200	30.6	228	28	92
	Christman	133	30.6	140	7	23
6. "	Authors	287	31.0	316	29	94
	Larson	318	31.0	343	25	81
	Christman	128	31.0	140	12	39
7. Rabbit	Authors	50.5	29.9	78.5	28	94
	Larson	80.0	29.9	104	24	80
	Christman	29.4				
8. "	Authors	27.1	30.0	56.2	29	97
	Larson	33.5	30.0	62.5	29	97
	Christman	17.8	30.0	34.4	17	55
9. "	Authors	69.5	29.7	100	31	102
	Larson	76.0	29.7	107	31	104
10. "	Authors	67.4	29.9	98.2	31	102
	Larson	135	29.9	166	31	103

averaged 98.4 per cent, by Larson's 101.4 per cent, by Christman's 62.2 per cent.

In the Larson procedure the urine is purified by treatment with phosphotungstic acid, basic lead acetate, and sulfuric acid prior

to the colorimetric estimation on the filtrate. We have applied this procedure to four specimens of dog urine and determined the allantoin in the filtrate by our technique. Such treatment gave results that were lower by about 14 per cent. This may be explained in part by the removal of urate and also by possible adsorption or occlusion of allantoin in the bulky precipitates obtained.

SUMMARY

A method for the colorimetric estimation of allantoin in urine has been described, based on the Rimini-Schryver reaction as applied to glyoxylic acid.

Some of the variables in this reaction have been studied and its specificity determined as applied to urine. By this method allantoin added to urine and in pure solution is estimated with an error of ±5 per cent and between 0.02 and 0.4 mg. is required for a determination.

A comparison has been made between the methods of Larson, Christman, and the authors on urine from dogs, cats, and rabbits both before and after the addition of allantoin. The Larson method gave values averaging 33 per cent higher than the authors' procedure; those by the Christman method were 35 per cent lower. Estimation of added allantoin averaged 98.4 per cent by the authors' method, 101.4 per cent by Larson's method, and 62.2 per cent by Christman's method.

We wish to express our thanks and indebtedness to the Banting Research Foundation for financial assistance to one of the authors (C. F. C.) during a part of this work.

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SPECTROPHOTOMETRIC STUDIES

IX. THE REACTION OF CYANIDE WITH NITROGENOUS DERIVATIVES OF FERRIPROTOPORPHYRIN*

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The purpose of this communication is mainly to call attention to a hitherto unrecognized or unappreciated reaction of CN^- with nitrogenous derivatives of ferriprotoporphyrin. CN^- has been found to form a complete combination with nitrogenous derivatives of ferriprotoporphyrin when added to solutions of the latter in concentrations as low as 0.1 mM per liter, equivalent to the concentration of hemin Fe present. This reaction of CN^- with nitrogenous derivatives of ferriprotoporphyrin must be distinguished from its reaction with ferri- or ferroprotoporphyrin itself. Earlier spectroscopic studies of Drabkin and Austin (3) and Barron (4) failed to make this distinction and require clarification. The possible relationship of the above reaction of CN^- with nitrogenous derivatives of ferriporphyrin to the inhibition by CN^- of cellular respiratory catalysis and to cyanide poisoning will be discussed.

Nomenclature

A new nomenclature for metalloporphyrin derivatives was proposed by the writer (1), because existing terms, such as *cyanide hemochromogen*, proved inadequate or ambiguous in describing the reactions in which the present interest was centered. Clark and associates (5) have independently adopted a similar nomen-

* Preliminary reports upon this work were presented before the meeting of the American Society of Biological Chemists at Baltimore, and at the Seventh Summer Conference on Spectroscopy, Cambridge, and abstracts of the reports have appeared (1, 2).

clature. In this nomenclature the various compounds are described as derivatives of ferri- or ferroporphyrin, or more specifically, ferri- or ferroprotoporphyrin, when the porphyrin involved is protoporphyrin. Thus, the compound variously designated as *cyanhematin*, *hematin dicyanide*, *cyanide parahematin*, and *oxidized cyanide hemochromogen* is now described by the term *dicyanide ferriprotoporphyrin*. In the writer's original proposal (1, 6) the compound or group coordinating with the iron porphyrin was written as cyano, pyridino, etc. A personal communication from Dr. W. M. Clark has suggested, however, that at present such a usage is inadvisable.[1]

Methods

For details of spectrophotometric technique, the reader is referred to the earlier publications of Drabkin and Austin (3, 8) and Drabkin (6). The hemin (ferriprotoporphyrin), from solutions of which the various derivatives were prepared, was isolated from washed dog erythrocytes by the method of Drabkin and Austin

[1] Shortly after the publication of the preliminary report (1) upon the proposed new nomenclature for derivatives of metalloporphyrins, Dr. M. L. Anson sent me a note with his own proposal for a new terminology for the heme pigments. This new terminology has now been published as a brief foot-note to one of Anson's papers (7). It is as follows:

$$\left.\begin{matrix}\textit{Hemo}\\ \textit{Hemi}\end{matrix}\right\}\textit{heme},\quad \left.\begin{matrix}\textit{hemochromogen}\\ \textit{hemichromogen}\end{matrix}\right\}\textit{hemechromogen},\quad \left.\begin{matrix}\textit{hemoglobin}\\ \textit{hemiglobin}\end{matrix}\right\}\textit{hemeglobin}$$

In a personal discussion of this question Anson agreed with me that his new proposal lacks desirable, general applicability. It is my opinion that Anson's proposal may be criticized also upon other grounds. (1) The introduction of new generic terms such as *hemechromogen* and *hemeglobin* appears unnecessary, as well as unusual from a chemical standpoint. According to Anson such terms would be useful only in general discussions involving no implications as to the state of iron. (2) The use of the prefix *hemi*, denoting ferric iron in Anson's proposal, seems unfortunate, since *hemi* has already an accepted chemical significance, as in, for example, hemiacetal, hemiterpene, and hemialbumose. In the above cases *hemi* denotes one-half. Under certain conditions hemoglobin is changed to a molecule with one-half the molecular weight of the original. In this instance, the term *hemiglobin* might denote such a change. In Anson's proposal hemiglobin is synonymous with methemoglobin or ferrihemoglobin. This is an indication of the type of confusion which may arise from the use of some of the new terms which Anson proposes.

(3). The iron content of the hemin, determined photometrically as ferrous *o*-phenanthroline by the writer's recently described procedure (9) was 8.41 per cent (theory, 8.57 per cent).

The values of ϵ (c = 1 mM per liter, d = 1 cm.) are reported upon an iron basis, 1 mM = 1 mM of Fe. The pH of most of the solutions upon which spectrophotometry was performed was determined by means of a glass electrode. The concentration of cyanide solutions, prepared by the solution of KCN in water, was determined both by titration with standard $AgNO_3$ and by titrating methemoglobin solutions with the CN^-, the latter reaction being followed spectrophotometrically. Both procedures yielded the same results. Details pertaining to the make-up of individual solutions reported appear in the legends accompanying the figures.

Results

Fig. 1 presents typical spectrophotometric data obtained in the reaction of CN^- with ferri- and ferroprotoporphyrin in alkaline solution, when different amounts of CN^- (see legend) were added to the hemin solutions. Spectrum Curves 1 and 3 represent respectively the partial and complete change of ferriprotoporphyrin to its dicyanide derivative (10). The spectrum of dicyanide ferriprotoporphyrin (Curve 3), is very similar both qualitatively and quantitatively to the spectrum of cyanmethemoglobin (8) and to the spectra (exemplified by Curve 3, Fig. 2) of the new cyanide derivative of nitrogenous compounds of ferriprotoporphyrin. In the presence of the reductant, $Na_2S_2O_4$, spectrum Curves 2 and 4 were obtained respectively from the solutions which had yielded Curves 1 and 3. Curves 2 and 4 correspond to the qualitative description of spectra given by Anson and Mirsky in their discovery of a stepwise reaction of CN^- with ferroprotoporphyrin (11). Spectrum Curves A, 1, and 3 do not lend themselves to analysis on the basis of the simultaneous addition of $2CN^-$ to ferriprotoporphyrin, as expected from Hogness, Zscheile, Sidwell, and Barron's careful study of this reaction (10). The present data suggest that stepwise addition of CN^- may occur with oxidized as well as reduced hemin, and call for further investigation.

The reactions of CN^- with ferri- and ferroprotoporphyrin, described above, differ strikingly from the reaction of CN^- with

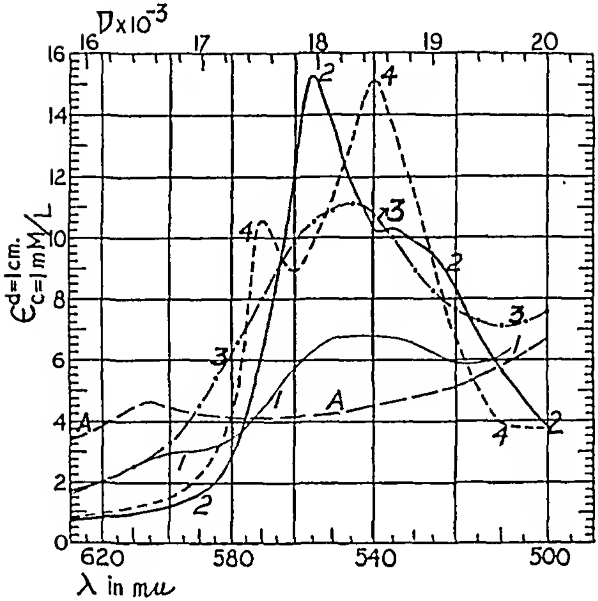

FIG. 1. Reactions of hemin (ferriprotoporphyrin) in alkaline solution with CN^-. Curve A, absorption spectrum of ferriprotoporphyrin in alkaline solution. The curve is based upon averages of several solutions, with hemin Fe concentration of 0.1 to 0.2 mM per liter and with pH varying from 9.9 to 12.2; there is but a negligible difference in spectrum in this range of pH. Curve 1, absorption spectrum of hemin plus a small amount of CN^-. Hemin Fe concentration, 0.0992 mM per liter; NaOH concentration, 0.00085 M; KCN concentration, 1.8 mM per liter; pH 10.92. Curve 2, absorption spectrum produced by the addition of $Na_2S_2O_4$ (final concentration of reducing agent 0.575 mM per liter) to the solution represented by Curve 1. Curve 3, absorption spectrum of dicyanide ferriprotoporphyrin (10), obtained by the addition of large amounts of CN^- to hemin in alkaline solution. Hemin Fe concentration, 0.1984 mM per liter; NaOH concentration, 0.00169 M; KCN concentration, 1080 mM per liter; pH 11.34. Essentially the same spectrum was obtained with hemin Fe concentration 0.0992 mM per liter, NaOH concentration 0.00085 M, KCN concentration 6.0 mM per liter. Curve 4, absorption spectrum of dicyanide ferroprotoporphyrin (11), produced by the addition of $Na_2S_2O_4$ (final concentration of reducing agent 0.575 mM per liter) to the solutions represented by Curve 3. The same spectrum was produced by adding to the solution represented by Curve 2 as little as 1.4 mM per liter of additional KCN, bringing the total KCN concentration to 3.2 mM per liter.

pyridine ferriprotoporphyrin, presented in Fig. 2. Upon the addition of 1 equivalent of CN^- per hemin Fe in solutions of pyridine ferriprotoporphyrin, a new cyanide derivative was

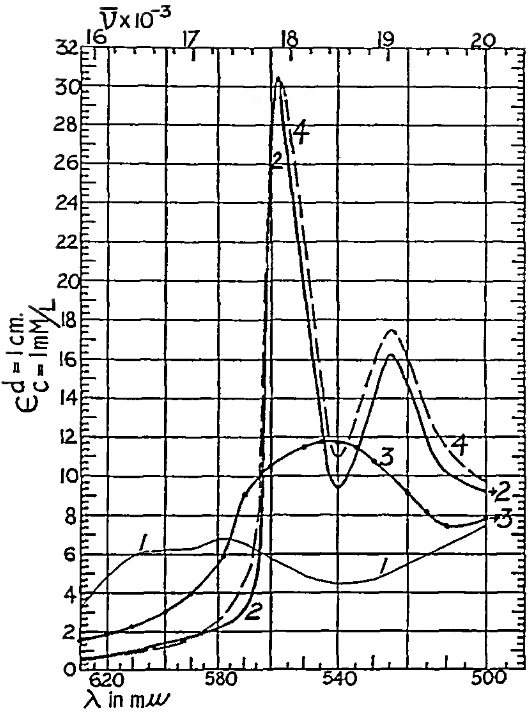

FIG. 2. Reactions of hemin (ferriprotoporphyrin) in alkaline solution with pyridine, and the reaction of the resulting nitrogenous derivatives with CN^-. Curve 1, the absorption spectrum of pyridine ferriprotoporphyrin. Hemin Fe concentration, 0.0992 mM per liter; NaOH concentration, 0.00085 M; pyridine concentration, 6330 mM per liter; pH 11.03. Curve 2, the absorption spectrum of pyridine ferroprotoporphyrin; the solution represented by Curve 1 with $Na_2S_2O_4$ added (final concentration of reducing agent 1.2 mM per liter). Curve 3, the absorption spectrum of the monocyanide derivative of pyridine ferriprotoporphyrin, produced from pyridine ferriprotoporphyrin by addition of 1 equivalent of CN^- per hemin Fe in solution. Hemin Fe concentration, 0.0992 mM per liter; NaOH concentration, 0.00085 M; pyridine concentration, 5060 mM per liter; KCN concentration, 0.1 mM per liter; pH 10.95. Curve 4, conversion of the cyanide pyridine ferriprotoporphyrin to a derivative with the spectrum of pyridine ferroprotoporphyrin by means of $Na_2S_2O_4$. Hemin Fe concentration, 0.0992 mM per liter; NaOH concentration, 0.00085 M; pyridine concentration, 4200 mM per liter; KCN concentration, 6.4 mM per liter (a 60-fold excess in comparison with the solution represented by Curve 3). To this solution, yielding the typical spectrum shown in Curve 3, $Na_2S_2O_4$ was added (final concentration of reducing agent 2.4 mM per liter).

produced, whose spectrum was typified by Curve 3, Fig. 2. It was established that complete reaction had occurred, under our conditions, with a ratio of CN^- to hemin Fe of 0.1 to 0.1 mM per liter. The addition of further small amounts of KCN, bringing the concentration of CN^- to approximately 10 mM per liter, produced no further alteration in spectrum. Analogous reactions with CN^-, which will not be presented here, were obtained with other nitrogenous derivatives of ferriprotoporphyrin and with so called oxidized globin hemochromogen. In the latter case, however, larger amounts of CN^- were required for complete reaction; namely, 6 equivalents of CN^- per equivalent of Fe.[2] The following points may be stressed concerning this apparently general reaction of CN^- with nitrogenous derivatives of ferriprotoporphyrin: (1) The spectra of the CN^- derivatives are similar to each other as well as similar to the spectrum of cyanmethemoglobin (8) and to the spectrum of dicyanide ferriprotoporphyrin (Curve 3, Fig. 1). (2) The conditions under which these CN^- derivatives were obtained are unusual. For example, Curve 3, Fig. 2, was obtained with a hemin Fe concentration of 0.1 mM per liter, and a ratio of CN^- to pyridine of 0.1 to 5000 mM per liter. Thus, in such mixed nitrogenous derivatives of ferriprotoporphyrin only minute concentrations of CN^- are necessary to form a tight combination in the presence of very large concentrations of other coordinating N compounds such as pyridine. Since only 1 equivalent of CN^- per hemin Fe was needed for complete reaction with the nitrogenous derivatives of ferriprotoporphyrin, it is deduced that a monocyanide derivative must have been produced. (3) The new monocyanide derivatives bear strong analogies to cyanmethemoglobin. The latter is formed from methemoglobin by the addition of 1 equivalent of CN^- per equivalent of Fe pres-

[2] With reference to reaction with CN^- the behavior of the naturally occurring hemochromogen, cytochrome *c*, appeared to be exceptional. The addition of large amounts of CN^- (600 mM per 0.1 mM of cytochrome iron) to solutions of oxidized cytochrome *c*, at pH 5.0 to 8.0, failed to produce any change in spectrum. This finding is in agreement with the recent report of Theorell and Åkesson (12), who have found, however, that a cyanide ferricytochrome *c* complex, spectroscopically similar to cyanmethemoglobin, may be produced, but only in strongly alkaline solutions of pH "not lower than 13."

ent. The analogy is supported further by the effect of the reducing agent, $Na_2S_2O_4$, in the two cases. With the loss of CN^- upon reduction, cyanmethemoglobin yields reduced hemoglobin (8). With the loss of CN^- upon reduction, the monocyanide derivatives of oxidized hemochromogens yield the corresponding reduced hemochromogens. This is the interpretation of Curve 4 in Fig. 2.

DISCUSSION

The general reaction of CN^- in very low concentration with oxidized hemochromogens, forming tight monocyanide derivatives of the latter, may be of interest from the standpoint of the mechanism of cyanide poisoning. Nitrogenous derivatives of ferriprotoporphyrin may be looked upon as prototypes of naturally occurring enzymes such as cytochrome oxidase. Cellular respiration involving the cytochrome system is inhibited by very low concentrations of cyanide (13). The very high affinity of CN^- for ferrihemochromogens suggests strongly that the inhibition of cellular respiration by cyanide may be accomplished through an analogous type of reaction.

SUMMARY

Spectrophotometric data are presented in support of the finding of a general reaction of CN^- with nitrogenous derivatives of ferriprotoporphyrin. In this reaction, as exemplified by the reaction of CN^- with pyridine ferriprotoporphyrin, 1 equivalent of CN^- per hemin Fe is sufficient to form a spectroscopically characteristic monocyanide derivative. The reaction is considered of interest from the standpoint of the mechanism of cyanide poisoning.

The monocyanide derivatives of oxidized hemochromogens appear to be analogues of cyanmethemoglobin. The spectroscopic data suggest that upon the addition of $Na_2S_2O_4$ to solutions of the monocyanide derivatives the corresponding reduced hemochromogens are obtained.

The spectra of the above monocyanide derivatives are also very similar to the spectrum of dicyanide ferriprotoporphyrin. The dicyanide derivative is obtained from hemin in the presence of large amounts of cyanide. It is distinguishable spectroscopically from the monocyanide derivatives by the fact that,

upon reduction, it yields so called reduced cyanide hemochromogen, or dicyanide ferroprotoporphyrin, which has a very characteristic spectrum.

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THE ACTION OF INTESTINAL NUCLEOPHOSPHATASE ON TOBACCO MOSAIC VIRUS

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Tobacco mosaic virus is a nucleoprotein having a molecular weight of approximately 5×10^7 (1). The nucleic acid, which comprises slightly more than 5 per cent of the virus, is of the ribose type (2) and is bound to virus protein in a mode of linkage as yet unknown. Investigation of tobacco mosaic virus structure by means of x-ray analysis (3) and electron microscopy (4) has not indicated a concentration of the comparatively dense nucleic acid in any particular part of the nucleoprotein molecule. The nucleic acid may therefore be uniformly distributed in the virus molecule. The many methods which have been devised to remove nucleic acid from virus protein are, in general, methods which result in loss of virus activity and disruption of the virus particle into much smaller protein fragments. These denaturation procedures include treatment with urea (5), alkali (6), acid (7), pressure (8), and heat in the presence of detergents.[1] Heat treatment in the presence of buffer results in the denaturation of the protein and liberation of the nucleic acid (7).

Of considerable interest, therefore, is a recent paper by Schramm (9) that deals with the action on tobacco mosaic virus of an intestinal nucleophosphatase preparation, first described by Klein (10). Certain experimental data obtained by use of this enzyme under conditions suggested by Bredereck and Müller (11) were interpreted by Schramm as indicating that the nucleic acid is completely removed from the nucleoprotein, leaving a protein whose size, homogeneity in the ultracentrifuge, and electrophoretic

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[1] Cohen, S. S., unpublished data.

mobility are the same as those of the original virus. The importance of this conclusion with respect to questions of virus and protein structure, as well as its uniqueness as compared to the results of other methods of investigation, required that an attempt be made to confirm this work.

The results obtained in the present investigation indicate that, under conditions approximating those described by Schramm, intestinal nucleophosphatase failed to remove nucleic acid from the virus. The activity of our enzyme preparation was established by its ability to hydrolyze both yeast and virus nucleic acid. An exact duplication of the experiments was made very difficult by the fact that the pH range stated to be necessary for the activity of the enzyme could not be attained under the conditions described. However, this pH range was achieved in other experiments. In addition, many variations of different parts of the experimental procedure, such as pH, time, age of virus preparation, etc., were made in attempts to secure conditions which might lead to the results described by Schramm. However, in no instance did exposure to the enzyme result in inactivation of the virus and separation of the nucleic acid.

EXPERIMENTAL

Preparation of Enzyme—Approximately 1 yard of intestine, immediately following the stomach, was removed from each of twenty freshly slaughtered calves. The intestines were cleansed with tap water and the intestinal mucosa scraped with a spatula. 925 cc. of 87 per cent glycerol were added to 186 gm. of mucosal brei and the mixture was shaken for 2 days at room temperature. 12 cc. of toluene were added and the enzyme preparation was stored at 4°. This procedure conforms in every detail to the modification of Klein's method as described by Schramm.

Activity of Enzyme on Yeast Nucleic Acid—It was considered necessary initially to establish the activity of the enzyme preparation towards a substrate described by Klein. Accordingly, the enzyme was tested on yeast nucleic acid (Merck) at three different hydrogen ion concentrations. The yeast nucleic acid was dissolved in 0.1 N NaOH to give a pH of 7.0 and the solution was filtered and used without further purification. The solution contained 1.70 mg. of P per cc. To 10.0 cc. of this substrate

were added 0.2 cc. of toluene, 2.7 cc. of 0.5 N veronal buffer of the desired pH, and 2.0 cc. of the enzyme solution. In the control experiments, water replaced the enzyme solution. The mixtures were incubated at 37° for 72 hours. At the start and at the end of the incubation period, 2.0 cc. samples were removed for pH determinations. These were measured by means of a glass electrode and the results are recorded in Table I.

To 10 cc. of each reaction mixture, 15.0 cc. of 10 per cent trichloroacetic acid were added at room temperature, and the mixture was kept at 4° for 1½ hours. The solution was filtered, and 0.5 cc. of 95 per cent alcohol were added to 9.5 cc. of filtrate. The mixture was kept at 4° for 24 hours. The supernatant liquid

TABLE I

Hydrolysis of Yeast Nucleic Acid (YNA) by Nucleophosphatase at Different Hydrogen Ion Concentrations

Preparation	pH at start	pH at end	Free P	Total free P in reaction mixture	Amount of hydrolysis
			mg. per cc.	*mg.*	*per cent*
YNA....................	7.0	6.9	0.46	17.8	100
Control.................	7.0	7.2	0.19	7.4	
YNA....................	8.0	7.7	0.45	17.5	100
Control.................	8.0	8.1	0.17	6.6	
YNA....................	9.0	8.1	0.46	17.8	100
Control.................	9.0	8.8	0.20	7.8	

obtained after centrifugation at 3000 R.P.M. for 15 minutes was examined for phosphorus by the King method (12). The results of these analyses are given in the fourth column of Table I. By the same method of analysis, 2 cc. of enzyme alone yielded 0.54 mg. of P and 10 cc. of the solution of yeast nucleic acid yielded 6.61 mg. of P. These figures correspond to the total of free and soluble phosphorus of the preparations under the conditions of the analytical method. From Table I, which summarizes the results, it can be seen that very little additional free phosphorus appeared in the control experiments, while all the phosphorus appeared in the free form in the system containing the enzyme. The experiments show that the enzyme preparation completely hydrolyzed yeast nucleic acid over the pH range of 7 to 9.

Activity of Enzyme on Virus Nucleic Acid—The preparation of virus nucleic acid used in the present studies was isolated by Dr. H. S. Loring by means of alkaline denaturation of tobacco mosaic virus (2) and was subsequently purified by dialysis. We have subjected this preparation of virus nucleic acid to ultracentrifugation, diffusion, and electrophoresis studies and found it to be quite homogeneous with a molecular weight of 11,000.[1] A description of these experiments will be published later in greater detail. A sample of this material was incubated with enzyme at pH 7.5 in 0.1 M veronal buffer for 96 hours. The experiment was carried out in the manner described previously for yeast nucleic acid. The results indicated a 96 per cent hydrolysis of this material by the enzyme preparation.

Action of Enzyme on Tobacco Mosaic Virus—The enzyme preparation was next tested on tobacco mosaic virus (TMV) under conditions exactly comparable to those described for the yeast nucleic acid. The virus solution was prepared from virus freshly isolated by differential centrifugation and contained 18.9 mg. per cc.

At the start and at the end of the incubation period, samples were removed for determination of pH. The solution was centrifuged for 20 minutes in an angle centrifuge at 3000 R.P.M. The slightly turbid supernatant fluid was decanted, and 10.0 cc. of this solution were sedimented in the quantity ultracentrifuge. This procedure was undertaken to recover all substances having a size comparable to that of tobacco mosaic virus, since the method of analysis for bound phosphorus described by Schramm was considered to be unsatisfactory.[2] The pellet obtained on ultracentrifugation was dissolved in water and the volume of the solution was adjusted to 10.0 cc. The solution was dialyzed overnight at 4° against distilled water and centrifuged at 3000 R.P.M. for 15 minutes. The supernatant liquid was used for determinations of

[2] It was found on attempting to use Schramm's procedure that the addition at room temperature (22°) of 30 per cent trichloroacetic acid to twice the volume of virus solution caused a liberation of 20 per cent of the phosphorus present in enzyme-treated and untreated virus preparations. The elevation of the temperature to 100° after addition of the trichloroacetic acid caused practically quantitative removal of the phosphorus from both types of protein preparations.

nitrogen, phosphorus, and biological activity. Nitrogen was determined by the Kjeldahl method. This value multiplied by the factor 6.25 was used to estimate the protein content of the preparation.

Biological activity was determined by the half leaf method (13) on *Phaseolus vulgaris* L., and a minimum of thirty-one leaves was used in each test. The enzyme-treated virus was compared to virus undergoing the control experiment. The virus concentrations used were approximately 2×10^{-5} gm. per cc.

The results, which are summarized in Table II, clearly show that, following exposure of tobacco mosaic virus to the nucleo-

TABLE II

Action of Nucleophosphatase on Tobacco Mosaic Virus (TMV) in Buffered Systems

Preparation	pH at start	pH at end	Virus recovered	Bound P	Virus P	Virus activity
			per cent	*mg. per cc.*	*per cent*	*per cent*
TMV..........	7.0	7.3	89	0.067	0.58	117
Control........	7.0	6.9	96	0.071	0.58	
TMV..........	8.0	7.9	95	0.061	0.50	97
Control........	8.0	7.9	93	0.064	0.54	
TMV*.........	9.0	8.5	81	0.055	0.53	76
Control*.......	9.0	8.7	66	0.046	0.54	

* The fact that tobacco mosaic virus is unstable in the vicinity of pH 9 probably accounts for the low yields and decreased activity of the nucleoprotein recovered after incubation at that pH.

phosphatase, practically all of the virus may be recovered and that the virus so recovered possesses the normal activity and phosphorus content. The experiments demonstrate that the virus is unaffected by conditions known to bring about the hydrolysis of free nucleic acid. It may be noted that ribonuclease brings about the hydrolysis of free virus nucleic acid but, although causing the reversible inactivation of tobacco mosaic virus, has no effect on the nucleic acid when bound to protein in the form of virus (14).

Activity of Enzyme on Tobacco Mosaic Virus in System without Added Buffer—In view of the results described above, it appeared desirable to repeat the experiments in the absence of added buffer in an attempt to approximate more nearly the conditions described

by Schramm. Two different virus preparations were used in these experiments. One containing 17.31 mg. per cc. was obtained by differential centrifugation; the second, containing 11.05 mg. per cc., was considerably older and had been purified by both $(NH_4)_2SO_4$ precipitation and differential centrifugation. The former, designated as TMV, was found to contain 0.49 per cent phosphorus, and the latter, designated as TMV-A, was found to contain 0.50 per cent phosphorus.

A series of four tubes was set up with each virus preparation. To 15.0 cc. of virus solution were added 2.0 cc. of the enzyme solution, 0.2 cc. of toluene, 4 drops of 0.1 per cent phenolphthalein, and sufficient 0.2 N NaOH (between 0.2 and 0.3 cc.) to bring the system to the phenolphthalein end-point. In some cases the end-points were deliberately exceeded in the hope that some variation in pH might be sufficient to produce the effects claimed by Schramm. However, the actual pH of all tubes was determined by means of a glass electrode before incubation for 72 hours at 37°, and one tube from each series was used to follow the hydrogen ion concentration of the system during the incubation period. After incubation the solutions were titrated with 0.02 N NaOH back to the phenolphthalein end-point. Within the period of incubation, the pH of the solutions apparently dropped from about 8.9 to about 8.3, since actual determinations by means of a glass electrode showed that TMV-1 dropped from pH 8.95 to 8.31 and TMV-A-3 from pH 8.89 to 8.32. Under these conditions, according to Schramm, the pH of the system should have fallen to between pH 7 and 8.

At the conclusion of the titration, all material possessing a size comparable to that of tobacco mosaic virus was isolated as described in the preceding experiment and analyzed for nitrogen and phosphorus. The results, summarized in Table III, clearly show that nucleic acid was not removed in significant amounts from either fresh or old virus nucleoprotein.

Four additional experiments carried out under the same conditions just described, except that an incubation period of 144 hours was used, showed substantially the same results. The percentage of phosphorus in the recovered sedimentable material was 0.54 for TMV and 0.50 for TMV-A.

Since under the conditions described by Schramm the pH range

of the reaction mixtures in the experiments described above apparently did not reach that which had been used by Bredereck and Müller, *i.e.* pH 7 to 8, a further test of enzymatic activity was made in the pH range 7.3 to 7.5 in the absence of added buffer. Two groups consisting of two tubes each were set up with TMV (18.9 mg. per cc.) and TMV-A (11.05 mg. per cc.). To 10.0 cc.

TABLE III

Activity of Nucleophosphatase on Tobacco Mosaic Virus over pH Range 8 to 9 in Absence of Added Buffer

Preparation	pH at start	Amount of 0.02 N NaOH added	pH at end	Virus recovered	Bound P	Virus P
		cc.		*per cent*	*mg. per cc.*	*per cent*
TMV-2	8.9	2.30	9.1	76	0.054	0.48
3	8.9	1.95	9.0	81	0.055	0.46
4	8.9	1.30	8.8	83	0.055	0.45
TMV-A-1	8.8	1.40	8.8	73	0.030	0.43
2	8.8	1.55	8.8	72	0.029	0.42
4	9.2	1.05	8.7	61	0.025	0.43

TABLE IV

Activity of Nucleophosphatase on Tobacco Mosaic Virus at pH 7.5

Preparation	pH at start	Amount of 0.02 N NaOH added	pH at end	Virus recovered	Bound P	Virus P
		cc.		*per cent*	*mg. per cc.*	*per cent*
TMV-1	7.50	0.20	7.36	75	0.047	0.46
2		0.30		81	0.061	0.46
TMV-A-1	7.50	0.35	7.30	69	0.022	0.55
2		0.68		64	0.036	0.53

of substrate were added 2.0 cc. of enzyme, 0.2 cc. of toluene, and 3 drops of 0.1 per cent brom-thymol blue. The mixture was then adjusted to the blue-green end-point with measured small amounts of 0.2 N HCl. A portion of one tube of each group was used to follow the pH variation of the system. The mixtures were incubated for 72 hours at 37°.

At the conclusion of the incubation period, sedimentable material was isolated and analyzed as described previously. The

with the protein, which is not removed unless dialysis against running water is adopted. The preparations here reported were dialyzed several days against running water, whereas dialysis against successive changes of distilled water was formerly employed.

TABLE I

Malonyl Serum Albumin Preparations

Carbohydrate-free crystalline horse serum albumin of McMeekin (3); pH of all experiments 7.5.

Experiment No.	CSA-6	CSA-7	CSA-9	CSA-10	CSA-11
Original amino N per gm. atom N, *mole*	0.080	0.080	0.080	0.087	0.081
Tyrosine content (4, 5) *per* gm. atom N, *mole*	0.023	0.023	0.023	0.023	0.023
Sum of reactive groups per gm. atom N, *mole*	0.103	0.103	0.103	0.110	0.104
C_3O_2 added per mole of reactive groups, *moles*	3.2	4.6	8.0	3.0	5.2
C_3O_2 added per gm. atom N, *mole*	0.33	0.47	0.82	0.33	0.54
Total combined malonyl per gm. atom N, *mole*	0.080	0.099	0.087		0.103
Ratio, final amino to total N	0.008	0.004	0.009	0.007	0.008
Amino N_2 blocked, *per cent*	90	96	93	92	91
O-Tyrosyl-combined malonyl per gm. atom N, *mole*		0.014*	0.020†	0.018‡	0.019†
Phenolic hydroxyl blocked, *per cent*		60*	88†	79‡	84†

* After 8 days at 2°.
† After 2 hours at 0°.
‡ After 18 hours dialysis.

Estimations of the extent of reaction with tyrosine were, however, quite erratic until it was appreciated that the phenolic ester linkage of malonic acid was labile in aqueous protein solution, undergoing a gradual hydrolysis. For these determinations, the procedures developed by Herriott (6), who employed Folin's phenol reagent, were found satisfactory. The pH 8 method gives the amount of free phenolic hydroxyl plus tryptophane in the malonylated protein, the pH 11 method the total content, the

difference representing the malonyl-bound tyrosine of the original protein. The average of fifteen determinations made by the pH 11 method on malonyl protein, 0.186 ± 0.002 mg. of tyrosine-tryptophane per mg. of nitrogen, agreed with the figure for untreated protein. This value equals 59 per cent of the sum of the best available figures for the tyrosine-tryptophane content of crystalline horse serum albumin, corroborating the figure reported by Herriott. This incomplete color development of tyrosine bound in protein is paralleled by a similar behavior of synthetic

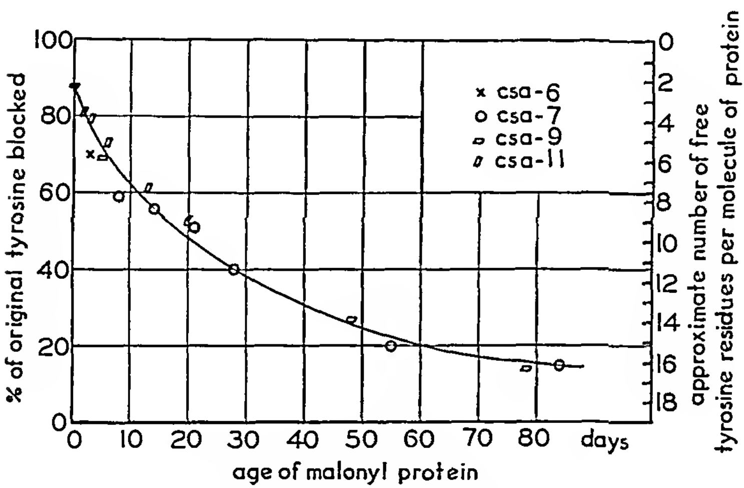

FIG. 1. Hydrolysis of O-malonyl group in aqueous solutions of malonyl horse serum albumin at 2° and pH 5.1. No buffer present.

tyrosine derivatives, which will be considered later in this paper. All figures for protein tyrosine have been corrected by this factor in order to give true values for the amino acid.

If malonyltyrosine was determined within 2 hours after treatment of the protein with C_3O_2, the solutions having been kept on ice meanwhile, almost 90 per cent of the total tyrosine was found to be covered. However, if salt-free solutions of the malonyl albumin of pH 5.2 were stored in the cold, rapid hydrolysis occurred and proceeded as shown in Fig. 1 where the analytical data for four preparations are assembled. After 9 to 12 months, a point was reached at which only 3 to 5 per cent of the tyrosine

was covered. Hydrolysis at 37° and pH 8.2 is much more rapid, being effectively complete in 2 days.

Carbon suboxide is unlike ketene in that at reactions between pH 7 and 8 it combines with both amino and phenolic groups at approximately the same rate. A solution of serum albumin was treated with an excess of C_3O_2, aliquots being removed at different stages during the addition to determine the extent of reaction with amino and phenolic groups. The results, plotted

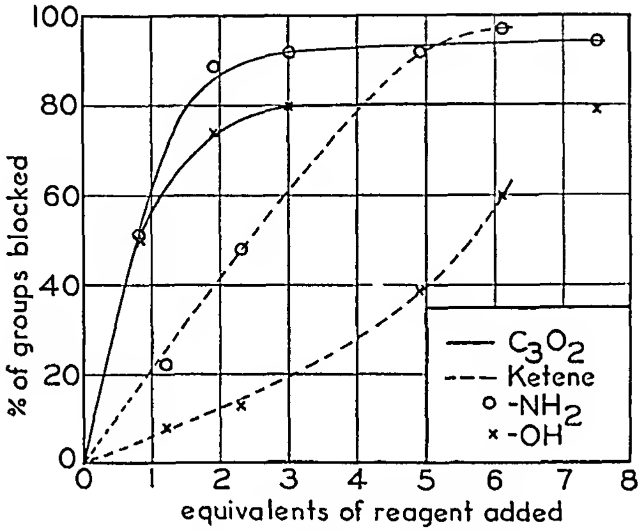

FIG. 2. Reactivities of C_3O_2 and ketene with the amino and tyrosine phenolic groups of serum albumin at 0°; pH of C_3O_2 experiments 7 to 8, that of ketene 6.5 to 7.5. The time required for the addition of 3.0 equivalents of C_3O_2 was 15 minutes; for 4.9 equivalents of ketene, 25 minutes. In calculation of the number of equivalents of reagent from the amount of base required to keep the pH constant, C_3O_2 was considered to react with the protein through only one of its functional groups.

in Fig. 2, are the basis for the above conclusion. In Fig. 2 we have used the experimental values for the per cent of total tyrosine and tryptophane actually covered by the reagent. Since these analyses were performed on samples which had been dialyzed for 18 hours to remove free malonic acid, the orginal combined malonic content should be several per cent higher (*cf.* Fig. 1). This factor and the further point that tyrosine makes up only 90 per cent of the amino acids, tyrosine and tryptophane, that react with the phenol reagent, lead us to conclude that the reaction

with phenolic groups is really complete when the curve levels off at 80 per cent.

A similar experiment with ketene indicates that it reacts much more readily with amino groups than with phenolic, but that the reaction of the latter approaches completion as excess reagent is

TABLE II

Phenol Tests of Synthetic Tyrosine Derivatives

Color tests were made by the methods of Herriott (4). The standard contained 0.30 mg. of tyrosine per ml. and concentrations of derivatives were chosen so that the equivalent of 0.30 mg. of tyrosine per ml. would be present.

Material	Concentration	Age of solution (2°)	Tyrosine calculated from color value		Per cent of theoretical	
			pH 8 method	pH 11 method	pH 8 method	pH 11 method
	mg. per ml.	*days*	*mg.*	*mg.*		
N-Chloroacetyltyrosine	0.426	Fresh	0.243	0.249	81	83
N-Carbobenzoxytyrosine	0.523	"	0.238	0.234	79	78
N-Carbobenzoxy-O-acetyltyrosine	0.596	"	±0	0.229	±0	76
Di-N-carbobenzoxytyrosyl malonate, Sample B	0.580	0*	0.123	0.223	41	74
		10*	0.133	0.223	44	74
		10 (25°)*	0.207	0.221	69	74
Tyrosine + Malonic acid	0.299 0.086	Fresh	0.296	0.295	99	99
Tyrosine + Malonyldiglycine	0.299 0.180	"	0.300	0.288	100	96
N-Carbobenzoxy-*l*-tyrosylglycine	0.617	"	0.262	0.262	87	87

* These solutions were prepared in 0.05 M phosphate buffer of pH 7.3 and were studied in this medium.

added. This confirms the results of earlier investigators using ketene (7, 8).

It was earlier mentioned that tyrosine and tryptophane bound in native proteins develop with the phenol reagent only 59 per cent of the color resulting when the test is made on a hydrolysate of the same protein. This suggests that the phenolic group does not

react so completely when the tyrosine of which it is a part is coupled in peptide formation. This idea has been tested with the N-substituted tyrosine derivatives shown in Table II, where it is seen that only 74 to 83 per cent of the theoretical color is developed. It was thought that N-carbobenzoxy-*l*-tyrosylglycine, having both the α-amino and carboxyl groups of tyrosine combined in peptide formation, might behave more like protein-bound tyrosine with the phenol reagent. It developed, however, 87 per cent of the theoretical color value, somewhat more than the N-substituted compounds. In general, then, substitution diminishes the intensity of the color developed by tyrosine with the phenol reagent, but this decrease cannot be correlated quantitatively with the number of substituent groups.

A sample of di-(N-carbobenzoxytyrosyl) malonate, prepared as earlier described (9), behaves like the other N-substituted tyrosine derivatives with the pH 11 method, giving 74 per cent of the theoretical color. With the pH 8 method, however, it gives an initial value of 41 per cent. We have not been able to obtain this compound in a satisfactory state of purity; on recrystallization it appears to undergo decomposition, possibly hydrolysis, with a shift in melting point, values ranging from 135–175° having been observed.

EXPERIMENTAL

The methods for adding C_3O_2 to proteins and for determining combined malonic acid are generally the same as already described (1). The use of C_3O_2 has, however, been greatly simplified by subjecting the crude material to several fractional distillations *in vacuo*. The purified material is stable over hydroquinone in the cold and may be stored in sealed glass tubes for several months without undergoing appreciable polymerization. The same technique was employed in the experiments with ketene.

Phenol Tests on Synthetic Tyrosine Derivatives. l-Tyrosine—As standards we have used two different samples from Hoffmann-La Roche, Nutley, New Jersey, and one, analytically pure, from Amino Acid Manufactures, Los Angeles. The last had earlier been found to be pure by ultraviolet spectroscopic examination.

N-Chloroacetyl-l-tyrosine—Hoffmann-La Roche; m.p. 152.5–153.5°.

N-Carbobenzoxy-l-tyrosine—Synthesis according to Bergmann and Zervas (10); m.p. 99–100°.

N-Carbobenzoxy-O-acetyl-l-tyrosine—From Mr. A. E. Barkdoll; according to Bergmann *et al.* (11); m.p. 120–122°.

Di-(N-carbobenzoxy-l-tyrosyl) Malonate—Synthesis according to Ross and Green (9). The observed melting points for two preparations were 168–174° with decomposition (Sample A) and 153–156° with decomposition (Sample B). The value earlier reported was 135° with decomposition.

Analysis—$C_{37}H_{34}O_{12}N_2$.	Calculated.	C 63.6, H 4.9
	Found (B).	" 62.9, " 5.0

N-Carbobenzoxy-l-tyrosylglycine—Synthesis according to Bergmann and Fruton (12); m.p. 98–101°.

DISCUSSION

For the purpose of comparing the total malonic acid introduced into the molecule with the total reactive groups present, we are basing our calculations on a figure of 70,000 for the molecular weight of horse serum albumin (13). Our protein was prepared by the method of McMeekin who reported a nitrogen content of 16.1 per cent for his product (3). There is good agreement regarding the tyrosine content, 4.67 per cent (4) or 4.79 per cent (5), corresponding to eighteen tyrosine residues per molecule. There is no analysis of the lysine content of this crystalline protein. However, an earlier analysis for serum albumin reported by Bergmann and Zervas in a review (14) gave 11.3 per cent, or 54 residues per molecule, as described above. The amino nitrogen content of Hewitt's crystalline carbohydrate-free protein (1.09 per cent) and that of our preparation (Table I) may be used to calculate the number of free amino groups per molecule. These are found to be 54 or 64, respectively, and represent the approximate number, primarily derived from lysine, with which the reagent might be expected to react. If these figures are used, there would be between 72 and 82 reactive amino and phenolic groups within the molecule.

Typical preparations of malonyl serum albumin were found to have 0.099, 0.087, and 0.103 moles of combined malonic acid per gm. atom of nitrogen (Table I). If from these analyses the

actual number of malonic acid residues per molecule of protein is calculated, figures of 80, 70, and 83 are obtained. These may be compared with the 72 to 82 reactive groups estimated above. Such a comparison supports our interpretation of the experimental data of Fig. 2, that 93 per cent reaction of the amino groups and 80 per cent reaction of the groups that reduce the phenol agent correspond to complete coverage of both lysine and tyrosine. This occurs after approximately 3 times the required amount of C_3O_2 has been added.

The addition of this amount of malonic acid to the serum albumin molecule will increase its molecular weight from 70,000 to about 77,000.

The observation that malonic acid bound to phenolic hydroxyl groups is not stable under physiological conditions is especially significant in view of the frequent use of ketene to determine the importance of tyrosine in physiologically active proteins. Although we have no evidence to prove that phenolic-bound acetyl is labile in this way, there is no reason to suppose that it would be far different. As yet we have noted only one experimental finding which might be attributed to such hydrolysis. Boor and Miller (15) observed that ketene reacts with gonococcus cells to inactivate them, and that only two out of six mice were killed by freshly acetylated cells, but after the preparation had stood a week, even in the cold, it was fatal to all six animals. It is possible that acetylation of tyrosine originally caused detoxication, and that gradual hydrolysis of these groups allowed the activity to return. That such an effect has not entered into the study of acetyl pepsin (7) is probably due to the fact that the digestive period is very short, 10 minutes, and the temperature low, 25°. The acetyl insulin of Stern and White (8) was tested by the mouse convulsion method, so that exposure to the enzymes and conditions existing in the body would be at a minimum. Further work with such physiological systems should, however, be carefully controlled to insure that such an unexpected hydrolysis does not occur.

SUMMARY

1. The free amino and tyrosine phenolic groups of serum albumin appear to have completely reacted with malonic acid after the addition of 3 times the theoretical amount of carbon suboxide.

2. Carbon suboxide, unlike ketene, reacts with free amino and tyrosine phenolic groups at approximately the same rate.

3. Malonic acid bound to tyrosyl phenolic groups is labile even in the cold and is quite rapidly hydrolyzed under physiological conditions.

4. Substituted tyrosine derivatives develop with the phenol reagent less than the theoretical intensity of color equivalent to their tyrosine content.

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LICHENIN AND ARABAN IN OATS (AVENA SATIVA)

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Like the other cereal grains, oat seeds contain several complex polysaccharides. Aside from starch, which makes up the greater part of the seed, there are levans (1), the two polysaccharides with which this paper deals, and presumably other as yet unidentified substances. In the course of work on cupric chloride crystallization patterns (2–4) it has been found that polysaccharides are frequently responsible for the specificity of the patterns of grain extracts. It has already been reported that this is true in the case of oats, and that the polysaccharide in question can be precipitated with alcohol (2) or acetic acid (4). The product obtained by precipitation with acetic acid seemed to contain less protein than that precipitated by alcohol.

The pattern-forming substance of oats has now been obtained in a state sufficiently pure for identification, and found to be identical with lichenin. In the course of the purification of this substance a very small amount of another polysaccharide has been isolated, though not in a pure state, which is believed to be an araban, with the properties of the araban described by Hirst and his coworkers (5–8).

The significant properties of authentic lichenin as compared with those of the corresponding oat preparation are as follows: Both give nearly identical patterns with cupric chloride crystals, both are readily soluble in hot water to give a solution which jells when it cools, and both are soluble in cold sodium hydroxide solution. The specific optical rotation of lichenin in 2 N sodium hydroxide is $[\alpha]_D = +8.3°$ (9), that of the oat preparation, $+6°$ ($\pm 1°$). The rotation[1] of the triacetyl derivative of lichenin in chloroform is

[1] All rotations mentioned are specific rotations for the sodium D line.

$-40.3°$ (10), while that of the preparation from oats is about $-37°$. The lichenin from oats when hydrolyzed gave products with a specific rotation of about $+47°$, from which glucosazone could be prepared. Total reducing sugars were found to be about 99 per cent of the product, of which 95 per cent is aldoses, while 1.3 per cent of the total was found to be pentose.

A comparison of the properties of Hirst's araban with those of the corresponding product from oats shows that both give remarkably high levorotation in water: araban $-160°$, oat product about $-140°$. The acetate of the Hirst preparation has a rotation of $-90°$ in acetone, that of the oat preparation $-105°$ in chloroform. An osazone of melting point 160° could be obtained from the hydrolysis products of the oat preparation, and the preparation gave the calculated yield of furfural when it was distilled with 12 per cent hydrochloric acid. The specific rotation of the hydrolysis products of the preparation was $+55°$ instead of $+105°$ as it should have been if araban were the only constituent. This discrepancy points to the presence of other polysaccharides as contaminants. Like the levans (*e.g.* (11, 12)) the oat araban is soluble in pyridine, and may be acetylated at room temperature with acetic anhydride and pyridine. This property facilitates its separation from lichenin.

The polysaccharides were separated from each other and from the rest of the soluble polysaccharides by fractional precipitation from water by acetic acid, followed by acetylation, and fractional precipitation of the acetates from ethyl acetate by an alcoholic solution of ammonium acetate. Incidentally, ammonium acetate is very valuable for polysaccharide work; frequently its addition to a colloidal suspension of a polysaccharide will bring about complete precipitation. In addition to this the salt will evaporate completely in the vacuum desiccator in the presence of sulfuric acid and sodium hydroxide.

Most of the methods given in the literature for the saponification of polysaccharide acetates (for example (12–15)) involve the action of an alkaline solution on a solid. To avoid this heterogeneous reaction the method of Kunz and Hudson (16) for the estimation of acetyl in sugar acetates has been adapted for use with polysaccharides. The polysaccharide acetate is dissolved in acetone, to which a limited amount of aqueous or alcoholic alkali can

be added without precipitation of the acetate. Precipitation takes place only when the insoluble free polysaccharide is formed. In this way quantitative saponification can be obtained in half an hour at room temperature. For volumetric analysis aqueous alkali should be used, since the presence of alcohol gives low results, presumably due to the formation of ester (*cf.* (15)).

EXPERIMENTAL

Preparation of Starch-Free Extract—500 gm. of Swedish Selected oats were ground to pass a 20 mesh screen and extracted three times with 2×10^{-4} N iodine solution (17). Each time the extraction mixture was allowed to stand for about an hour, then strained through heavy muslin. The combined extracts, about 2 liters, were centrifuged to remove most of the starch, and then 100 gm. of "celite analytical filter aid" (Johns-Manville) were added. The mixture was filtered with suction through a layer of celite, to give a crystal-clear yellow filtrate.

Earlier attempts to prepare a starch-free extract by simple centrifugation, filtration through paper pulp, etc., were uniformly unsuccessful. Centrifugation yielded solutions that were always faintly opalescent, while filtration through paper pulp was too slow, and much of the desired material was lost through adsorption. If the preparation of the clear extract took more than 8 hours, serious losses resulted.

Preparation of Crude Polysaccharides—To the solution were added 250 cc. of 40 per cent lead acetate solution, and 10 cc. of 50 per cent acetic acid. A light precipitate separated, leaving an opalescent solution which was clarified by filtration through celite, after which 150 cc. of concentrated ammonium hydroxide were added, which caused the separation of a dense white gelatinous precipitate. The precipitate was collected in the centrifuge as a thick cream, and the lead removed as the carbonate with carbon dioxide. When an attempt was made to concentrate the resulting solution *in vacuo* at 60°, it foamed violently, and some precipitate formed. The solution was therefore heated to 95° to coagulate proteins, and filtered hot without suction. The clear filtrate clouded when it cooled, and could not be clarified with celite. The cloudiness consisted presumably of a heat-stable protein, possibly like Osborne's Preparation 20 (18).

To the cloudy solution, about 750 cc., were added 15 cc. of glacial acetic acid and 8 liters of 95 per cent alcohol.[2] A heavy white precipitate separated which was filtered with suction, leaving a yellow filtrate. The precipitate was washed with alcohol and ether. It had a light buff color when moist, and weighed, dry, about 9 gm. This preparation consisted principally of polysaccharides, but contained considerable protein.

Preliminary Fractionation of Polysaccharides—The precipitate just described was added to 50 cc. of water. The powder gradually swelled to form a thick syrup which was heated on the water bath. After it had been cooled to room temperature, 50 cc. of glacial acetic acid were added. At the end of 2 days the whole had set to a jelly, which was centrifuged, and about 50 cc. of clear supernatant solution were obtained. The jelly was extracted six times, each time with 50 cc. of 50 per cent acetic acid, then an equal volume of alcohol was added to it, and the precipitate was collected, washed with alcohol, and dried. This 50 per cent acetic acid-insoluble material weighed 4 gm., or slightly less than 1 per cent of the oats. A sample, dissolved in 2 N sodium hydroxide and centrifuged, gave a light brown solution which showed a specific rotation of about +8°. The further treatment of this fraction is described later, under "Purification and properties of lichenin."

The combined supernatant solutions obtained in the foregoing extraction were clarified by filtration through celite. To the filtrate, 300 cc., were added 15 gm. of ammonium acetate and 1200 cc. of alcohol. A brown precipitate separated that weighed, dry, 2.7 gm. This was dissolved in 30 cc. of water, 0.5 gm. of ammonium acetate was added, and the mixture boiled. A syrupy solution resulted which was filtered through celite. Filtrate and washings came to 45 cc. Enough 97 per cent acetic acid was added to make the acetic acid concentration 75 per cent, and a precipitate formed which settled readily. The precipitate was separated by centrifugation and was washed with 75 per cent acetic acid. This fraction weighed 0.75 gm. and had a specific rotation of −42° in water.

Glacial acetic acid was added to the supernatant solution until the concentration of acid was 88 per cent. The precipitate which

[2] The alcohol used throughout this work contained 5 per cent of methyl alcohol.

formed was collected and washed with acetic acid of the same strength. This fraction weighed 0.98 gm. and had a specific rotation of $-41°$ in water.

Separation of the Highly Levorotatory Fraction—The 88 per cent acetic acid-insoluble fraction was dissolved in water and precipitated with 9 volumes of alcohol. The precipitate could then be almost entirely dissolved in 15 cc. of pyridine at 50° (*cf.* (19)), though a little gelatinous residue remained undissolved. The mixture was cooled to 25°, and 15 cc. of acetic anhydride were added with cooling. A heavy precipitate formed at once and dissolved in the course of the next half hour. After 24 hours at room temperature the acetylation mixture was added to an excess of cold water. A brownish gum separated, which was collected, dissolved in a little acetone, and precipitated with a large excess of alcohol. The acetate weighed 1.02 gm.

The 75 per cent acetic acid-insoluble fraction was acetylated in the same way. The acetylation mixture did not become clear at room temperature even after several days, and was therefore filtered through celite to give a clear colorless filtrate. When this was added to water, a colloidal suspension resulted, which could be coagulated by the addition of ammonium acetate. This polysaccharide acetate fraction weighed 0.72 gm.

Neither of these acetates dissolved to give a clear solution in chloroform. Accordingly each was taken up in 5 cc. of pyridine together with 5 cc. of acetic anhydride, allowed to stand overnight at room temperature, and again precipitated. The resulting preparations were entirely soluble in chloroform. Their specific rotations in chloroform were $-57°$ for the 75 per cent acetic acid-insoluble preparation, and $-44°$ for the 88 per cent acid-insoluble fraction.

Each acetate was dissolved in 12 cc. of ethyl acetate, and 15 cc. of 1 per cent ammonium acetate solution in alcohol were gradually added. Gummy precipitates resulted, which were collected by centrifugation as syrups. The syrups were redissolved and reprecipitated twice more with the same volumes of solvents. The final supernatant solutions contained nothing that could be precipitated by alcohol. The precipitates were rubbed up with alcohol, filtered, and dried. The supernatant solutions were not further investigated.

By this procedure, the 75 per cent acid-insoluble fraction yielded 180 mg. of a preparation whose rotation was $-105°$ in chloroform, and the 88 per cent acid-insoluble, 30 mg. of a preparation whose rotation was $-92°$. These were combined. It was found possible, by repeated solution of the material in ethyl acetate and precipitation with decreasing amounts of alcohol, to separate about 12 mg. of a fraction with a rotation of $-84°$, while the rotation of the remainder was $-105°$. Further attempts to fractionate the latter, either with ethyl acetate and alcohol, or with chloroform and petroleum ether, yielded only fractions with a rotation of $-105°$.

Properties of Levorotatory Fraction—Samples of the acetate weighing 25 and 44 mg. were analyzed for acetyl content by the method outlined earlier in this paper. They were dissolved in 10 and 15 cc. of acetone, respectively, and 2 and 3 cc. of 0.2 N sodium hydroxide were added. After half an hour water was added, and the excess alkali titrated with 0.02 N hydrochloric acid to pH 8, with the use of thymol blue as an indicator. Found, 40.0 and 40.4 per cent CH_3CO. The free polysaccharide was recovered from the titration mixtures and reacetylated with acetic anhydride and pyridine at 100° rather than at room temperature, as had been done earlier. The acetate so prepared weighed 59 mg. Its rotation was $-105°$, and analysis showed 40.7 and 41.1 per cent CH_3CO (calculated for $C_5H_6O_4(COCH_3)_2$, 39.8 per cent CH_3CO).

The remainder of the acetate was saponified at room temperature by the addition of a slight excess of 0.5 N alcoholic potassium hydroxide to its solution in acetone. The free polysaccharide separated almost immediately as a brittle solid that filled the whole solution. This gradually crumbled and settled. It was dissolved in water and precipitated with ammonium acetate in alcohol. Two determinations of optical rotation in water (one after recovery from reacetylation) gave $-146°$ and $-136°$; mean $-141°$.

2.920 mg. of the polysaccharide were analyzed for carbon and hydrogen.[3]

$C_5H_8O_4$. Calculated, C 45.5, H 6.06; found, C 44.16, H 6.13

An 11.8 mg. sample of the polysaccharide was hydrolyzed with 0.67 N hydrochloric acid for 3 hours at 100°. Reducing sugars

[3] By the laboratory of Dr. Carl Tiedcke, New York.

were determined on one-tenth aliquots by the method of Hanes (20). Found, 1.39, 1.33, 1.29, 1.32 mg., calculated as glucose or arabinose; theoretical for $C_5H_8O_4$, 1.34 mg. Hydrolysis with 2 N hydrochloric acid gave results about 10 per cent below the theoretical.

Reducing sugars were determined in the polysaccharide itself by the micromethod of Folin for the estimation of blood sugar (21). Found, 0.7 per cent, calculated as glucose.

A sample weighing 19.4 mg. was hydrolyzed as above, and the rotation of the hydrolysis products in a volume of 3 cc. was determined. The specific rotation was +55°. 0.5 cc. of this solution was neutralized, and treated with phenylhydrazine hydrochloride and sodium acetate. A crystalline osazone separated after the mixture had been heated to 100° for some time. The crystals were very similar in appearance to those of glucosazone. Attempts to recrystallize them from 50 per cent alcohol were unsuccessful; the alcoholic solution was therefore extracted with ether, and the osazone precipitated from the latter in several fractions with petroleum ether. The two least soluble fractions melted at 160°. This is the melting point of arabinosazone.

3.18 mg. of the polysaccharide were distilled with 12 per cent hydrochloric acid by a micro modification of the method of Kroeber (22). 2.75 mg. of phloroglucide were obtained, from a distillate volume of 40 cc. This is equivalent to 3.20 mg. of araban.

The polysaccharide was a white powder which was very hygroscopic, but not usually deliquescent. It dissolved very readily in water, from which it could be precipitated by large volumes of alcohol, acetone, etc., particularly if a little ammonium acetate was present. When freshly precipitated, it was readily soluble in pyridine.

Purification and Properties of Lichenin—The 4 gm. of 50 per cent acetic acid-insoluble material were added to 20 cc. of water at 100° to give a light brown turbid suspension, and precipitated with 80 cc. of 1 per cent ammonium acetate solution in alcohol. The precipitate was washed once with alcohol, and 20 cc. of pyridine were added. The mixture was triturated thoroughly in the cold, centrifuged, and the supernatant solution, which contained some alcohol-precipitable material, was discarded. 20 cc. more pyridine were added to the precipitate, which was then acetylated at 100° for 5 hours with 20 cc. of acetic anhydride. A considerable amount

of insoluble material was centrifuged off, and the acetate precipitated from the solution with cold water. It weighed about 6 gm.

The crude acetate was dissolved in ethyl acetate to form a turbid solution. This was clarified by filtration through celite. 50 cc. of 1 per cent ammonium acetate in alcohol were slowly added to the 45 cc. of ethyl acetate solution, and the mixture was centrifuged. Two clear liquid layers formed, the lower one a thick syrup. The supernatant solution was poured off, and the syrup redissolved in 20 cc. of ethyl acetate and precipitated with 23 cc. of alcoholic ammonium acetate. This process was repeated with 10 cc. of ethyl acetate and 12 cc. of alcohol. The syrup finally obtained was triturated with alcohol and dried. This fraction (Fraction I) weighed about 1 gm. It was not further investigated. To the combined supernatant solutions were added 10 cc. of alcohol, and, as before, a syrup was obtained, which was redissolved and reprecipitated to yield Fraction II. The supernatant solutions from this gave Fractions III and IV in the same way. A little material remained dissolved in the final supernatant solution, and was discarded. The properties of Fractions II, III, and IV are shown in Table I.

TABLE I

Properties of Oat Lichenin Fractions

	Fraction II	Fraction III	Fraction IV
Weight, *gm*.	1.4	2.3	0.8
Rotation of acetate in chloroform, *degrees*	−39	−36.2	−36.6
" " free polysaccharide* in 2 N NaOH, *degrees*	+6	+5	+7
Rotation of hydrolysis products,† *degrees*	+48	+49	+46.5
Sugar found after hydrolysis (as glucose), *per cent*	99.2	98.5	100.0

* Obtained by saponification exactly as was the araban.

† After hydrolysis with 0.67 N HCl for 3 hours at 100°.

The following data in addition to those given in Table I were determined for Fraction III. Its acetyl content was 44.6 per cent (calculated for $C_6H_7O_5(COCH_3)_3$, 44.8). The acetate was saponified, and pentosans were determined in 150 mg. of the free poly-

saccharide; found, 1.3 per cent pentosans. A sample of the polysaccharide was hydrolyzed with 0.67 N hydrochloric acid, and aldoses were determined in the hydrolysate by the method of Cajori (23). 94.4 per cent of aldoses were found, calculated as hexose. The difference between this figure and that of 98.5 per cent for the total sugars formed on hydrolysis (Table I) represents about 4 per cent of ketose in this fraction. An osazone was prepared from another sample of the hydrolyzed polysaccharide. It melted at 206–208° after it had been recrystallized from 50 per cent alcohol, and it had the typical appearance of glucosazone.

The polysaccharide was a white powder, nearly insoluble in cold water, but readily soluble in hot. This solution set to a jelly when it was cooled. The preparation dissolved to give a clear solution in cold 2 N sodium hydroxide. It was hygroscopic, but not deliquescent.

Cupric chloride crystal patterns were determined as described previously (2). The patterns of the oat lichenin were nearly identical with those of lichenin prepared from Iceland moss (*Cetraria islandica*). This was true whether the polysaccharides were used alone, or whether a trace of protein was added to each, though the protein markedly altered the patterns. Photographs of the oat lichenin patterns have been shown in a recent paper (4), where they were labeled merely "oat polysaccharide."

DISCUSSION

Karrer and his coworkers in 1924 (24–26) suggested that the name "reserve cellulose," apparently rather loosely used at that time, be restricted to lichenin. They preferred to use the name reserve cellulose since they believed that the polysaccharide was much more widely distributed in nature than it had previously been thought to be. This belief was based on the demonstration that lichenase could be found in the seeds of most plants, and it was reasoned that the enzyme would not occur without its substrate. Lichenin has not until now, however, been actually isolated from the higher plants. The fact that it can be obtained from oats bears out Karrer's hypothesis, and suggests that it actually serves as a cellulose reserve in the oat seeds.

The presence of araban in the oats is perhaps of only academic interest. The arabans are wide spread in nature, being a part of

the pectin complex (6). It is believed, however, that this is the first time an araban has been found in the grain seeds.

The methods used in this investigation, fractionation of the free polysaccharides with acetic acid and fractionation of the acetates, together with the application of the cupric chloride crystallization patterns where possible in addition to the more common means of identification, may prove to be of value in the separation of other polysaccharide mixtures.

SUMMARY

Two polysaccharides, identified as lichenin and araban, have been obtained from oat seeds.

Lichenin is the polysaccharide of oats responsible for its cupric chloride crystallization pattern. Its isolation tends to confirm a hypothesis of Karrer and his coworkers that lichenin is wide spread in nature as reserve cellulose.

A convenient method is described for the saponification of polysaccharide acetates and determination of their acetyl content.

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THE CARBON DIOXIDE TENSION AND ACID-BASE BALANCE OF JEJUNAL SECRETIONS IN MAN

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The comparative inaccessibility of the human small intestine accounts in part for the notable scarcity of information concerning the composition of its contents, particularly with respect to its acid-base balance. Occasional observations on secretions obtained through jejunal fistulas following necessary abdominal surgery have afforded some information on the physical and chemical characteristics of mixed intestinal juices. The bulk of scientific information regarding the chemical composition and properties of jejunal juice, however, has largely come from observations made on laboratory animals. The demonstration of a practical method (1, 2) for intestinal intubation in man has provided a means of obtaining intestinal secretions without recourse to surgical procedures. It is the purpose of the present paper to present (*a*) data on the acid-base balance of human jejunal juice, (*b*) the limits of normal variation, and (*c*) the rate at which the acid-base balance of the juice returns to normal after the administration of acids and alkalies. In the study of Karr and Abbott (3), on the acid-base balance of the intestinal secretions, a few observations on the pH and bicarbonate of jejunal juice were reported. Our data extend these observations to include calculations of the CO_2 tension and to describe in greater detail the response made by the acid-base balance to displacement from normal.

The jejunal juice was obtained from men and women through intestinal intubation by the technique described by Miller and Abbott (1, 2). A triple lumen tube was used, with a single bag for part of the work, and a double bag (to isolate a portion of the small intestine) for the remainder of the investigations. Fig. 1 shows in diagram the intestinal end of the two types of tubes required.

The results of forty-seven successful intubations made on thirty subjects furnish the data in this report. Thirteen of these intubations were on individuals with achlorhydria. Of the thirty subjects, three had four separate intubations each, one had three, six had two, and twenty had but one intubation. While the tube was in place in a given subject, several samples of jejunal secretions were withdrawn for a series of observations. The youngest subject was 17 years and the oldest 58 years of age. Twenty-one of the subjects were males (thirty-eight intubations) and eight were females (nine intubations).

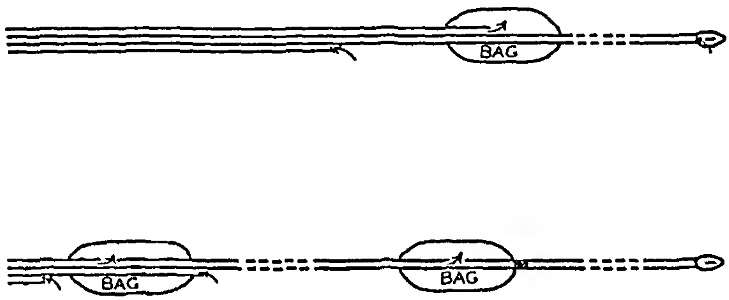

FIG. 1. Diagram of tubes used in procuring intestinal secretions

Procedure for Withdrawal of Fluids

All intubations were made after a 15 hour fast. In many instances, the first attempt at intubation consisted simply of teaching the subject how to swallow the tube. When the tube reached the stomach, during such an initial attempt, it was allowed to remain for an hour or more, primarily to accustom the subject to the presence of the tube. 1 or more weeks later, the process was repeated, with an effort to manipulate the tip of the tube into the duodenum, for which a fluoroscope and pressure on the abdominal wall, described by Morgenstern (4), were employed. This led to passage of the deflated, thin walled latex bag into the duodenum in 75 per cent of the trials. Once the bag was well past the pylorus (preferably well into the second portion of the duodenum), it was inflated with air. When two bags were on the tube, it was found that both must be well into the duodenum before their inflation. Usually, the jejunum was reached a half hour or so after inflation of the bag.

During the several hours of experimental observations following each successful intubation, an effort was made to keep the tip of the tube in the upper or midjejunum. This necessitated an occasional reexamination under the fluoroscope. At times the bag had to be deflated and the tube partially withdrawn because motor movements of the small bowel had moved several feet of intestine above the bag. The subjects were instructed, furthermore, not to allow the tube to be swallowed past a given mark. Although one cannot be sure of the exact location of the end of the intestinal tube during prolonged experiments, it is believed that most, if not all, of the intestinal fluid examined in this report was from the jejunum. In the observations on juice obtained from isolated intestinal loops, only jejunal juice was obtained; in those experiments in which the tube carried a single bag, the jejunal juice may have included salivary, gastric, and pancreatic secretions.

Analyses

The specimens were collected under oil in a closed system with constant suction from a 30 inch water column siphon. Carbon dioxide content was determined by the manometric method of Van Slyke and Neill (5). The pH of the juice was determined colorimetrically with the bicolor colorimeter of Hastings (6), and in some instances also electrometrically with the Leeds and Northrup glass electrode potentiometer. Phenol red (range 6.6 to 7.8 pH) and chlor-phenol red (range 5.0 to 6.8 pH) were suitable indicators for the pH variations of most of the specimens of jejunal juice (7).

Total base was determined by the method of Fiske (8). Chlorides were determined by the method of Wilson and Ball (9). The concentration of osmotically active solutes was estimated from the freezing point depression. The analyses reported by Karr and Abbott (3) indicate that "the only significant osmotically active solutes in the fasting intestinal contents are the chloride and bicarbonate salts." Confirmation of this was found by comparing the osmotic pressure of jejunal juice as calculated from (*a*) freezing point lowering, (*b*) total base determination, and (*c*) summation of chloride and bicarbonate present. (These data will be presented in detail in a subsequent publication.)

Calculations

The colorimetric pH was determined at room temperature and corrected to its value at 38° by subtracting 0.006 pH per degree. The concentration of HCO_3 and the CO_2 tension were calculated from the Henderson-Hasselbalch equation, values being assigned for pK′ and α_{CO_2} appropriate to the salt concentration of each specimen of juice. The value of pK′ was calculated from the equation of Hastings and Sendroy (10), $pK' = 6.32 - 0.5 \sqrt{(B)}$, where (B) is the concentration of total base in equivalents per kilo of water. The value of the solubility coefficient for carbon dioxide, α_{CO_2}, was calculated from the equation $\alpha_{CO_2} = 0.546 - 0.109$ (B), derived from the data on the solubility of CO_2 reported by Van Slyke, Sendroy, Hastings, and Neill (11).

Total Base

The total base content of the secretions of the upper small intestine, obtained by fistula from man, cat, or dog, has been reported to be approximately 160 to 170 milliequivalents per liter (12, 13). With intestinal secretions obtained by intubation in man, Karr and Abbott (3) found rather wide variations of total base, with many results indicating a hypotonicity as compared to the normal osmolar concentration of solutes of blood plasma. In our experience, the lower values of total base in jejunal juice were usually found only in the first specimens removed after the tube had passed the duodenojejunal flexure, and probably represent dilution of jejunal juice with base-poor secretions such as saliva and gastric juice. Of forty-one such "first specimens" in our series, the total base values were from 110 to 170 (average 139) milliequivalents per liter. Samples of fasting jejunal secretion taken subsequent to the first specimen were analyzed in twenty-two instances. The lowest total base value in this particular group was 144, the highest 170, with an average figure of 158.4 milliequivalents per liter. This compares favorably with the usual values for total base of normal serum and suggests that jejunal juice is normally isotonic with body fluids. The distribution of the total base values of the first and second specimens is shown in Fig. 2.[1]

[1] The actual experimental data from which Figs. 2 to 7 have been prepared may be obtained by applying to the authors.

Acid-Base Balance

Normal Variations—The data on the pH, HCO_3, and CO_2 tension of the samples of juice from normal fasting subjects and from patients with achlorhydria are presented in Fig. 3. These data are plotted logarithmically in a manner similar to that employed for urine by Sendroy, Seelig, and Van Slyke (14). The grouping of the results suggests that human jejunal juice tends, within certain limits, to have a characteristic acid-base balance, as indicated by the concentration of the points within the area limited by the heavy lines. It will be seen that, except in a few isolated instances, the pH values were between 6.8 and

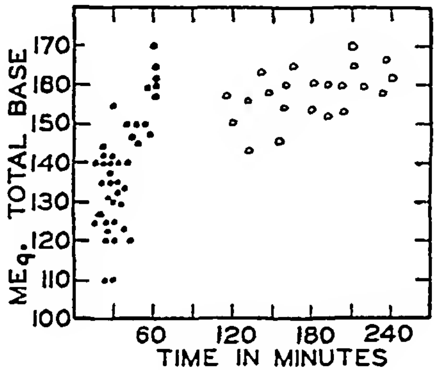

FIG. 2. Relation of total base content to the time of removal of fasting jejunal juice. ● designates initial specimen; ○ later specimen.

6.2, and the CO_2 tension between 60 mm. and 200 mm. Since most of the points had CO_2 tensions below 150 mm., this value is arbitrarily chosen as the upper normal limit of CO_2 tension.

The acid-base balance of normal jejunal juice may, therefore, be defined as having an average pH of 6.5 ± 0.3 and an average CO_2 tension of 100 ± 50 mm. Under these conditions, the HCO_3 concentration may vary from 2 to 10 mM per liter at a CO_2 tension of 60 mm. to values from 6 to 20 mM per liter at 150 mm. It was found in three instances, indicated by the lines joining several points, that successive samples drawn from isolated intestinal segments tended, with time, to have acid-base values within the normal area, though the initial samples in two instances had values well outside this area. This substantiates the

hypothesis that the acid-base balance of the juice tends to be maintained within certain restricted limits.

Since the acid-base values of samples of juice from patients with proved achlorhydria are randomly distributed among the values from normal individuals, the possibility that the high CO_2 tension of the juice is the result of contamination by the HCl of gastric juice is precluded. The composition of the juice, with

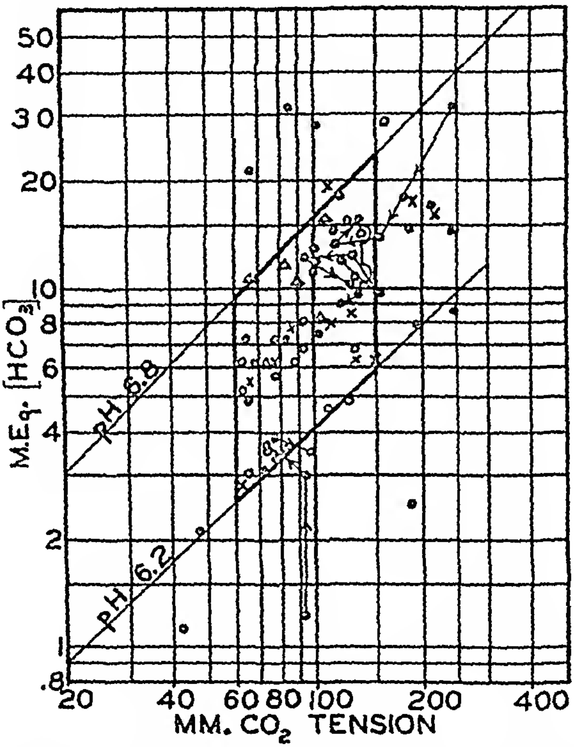

Fig. 3. The heavy lines demarcate the normal acid-base area. ●, ×, denote acid-base balance of jejunal juice from subjects with normal gastric secretion; ○, △ from subjects with proved achlorhydria.

respect to its acid-base balance, corresponds to that which would result if about 15 mM of HCl per liter were added to an ultrafiltrate of blood plasma. Had the increase in CO_2 tension been due to metabolic CO_2, it is difficult to understand why it was not more nearly equilibrated with the CO_2 tension of blood. In view of these considerations, it is concluded that the high CO_2 tension of jejunal juice is the result of the specific secretory processes by which the juice is formed. The present results on the acid-base

balance of the jejunal juice of man are consistent with the observations made by Robinson (15) and by Herrin (16) on Thiry-Vella jejunal loops in dogs.

Effect of HCl—The effect of the instillation of 100 cc. of 0.1 N HCl into the jejunum on the acid-base balance of the juice was

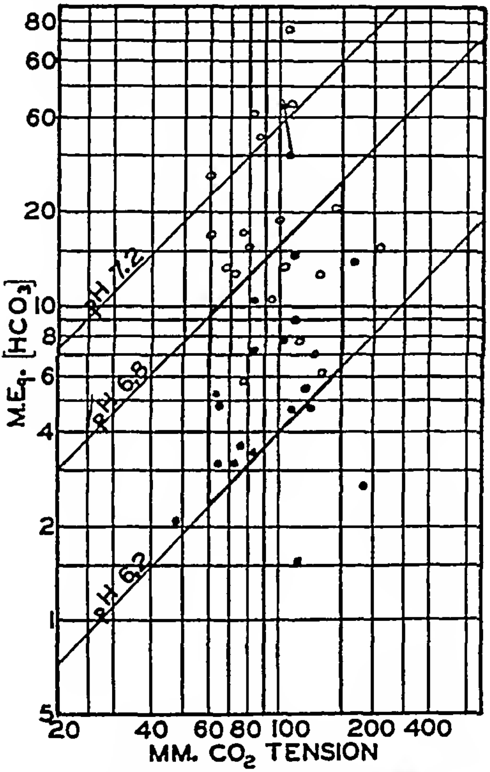

Fig. 4. Acid-base balance of jejunal juice before, ●, and 1 hour after the instillation of 100 cc. of 0.1 N hydrochloric acid, O.

studied in two series of experiments. In the first series, samples of juice were obtained before and 1 hour after the instillation of the acid; in the second series, the samples of juice were obtained at approximately 10 minute intervals during the 1st hour after giving the HCl. The results of these observations are shown in Figs. 4 and 5.

It is seen from the data of Fig. 4 that there is a definite tendency for the juice to have a higher HCO_3 concentration and higher pH

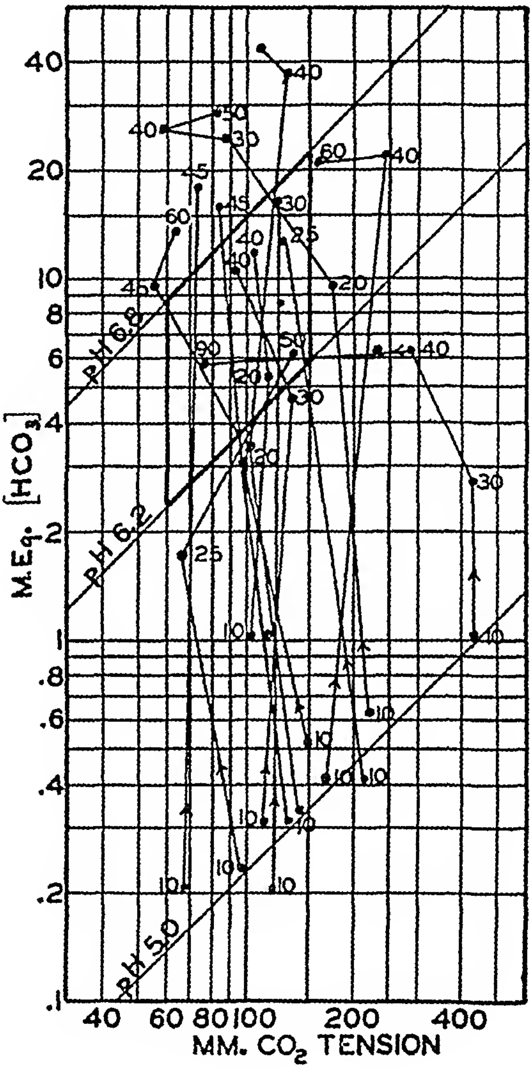

FIG. 5. Changes in acid-base balance following instillation of 100 cc. of 0.1 N HCl into the jejunum. The figures denote the time in minutes after the instillation of the HCl that the specimen was obtained.

1 hour after the administration of HCl. The CO_2 tension remains within normal limits, however. This change in the acid-base balance of the juice toward the alkaline side is no doubt the result

of secretin liberation and the resultant secretion of pancreatic juice and bile.

The data of the second series of twelve observations, plotted in Fig. 5, show even more strikingly the nature of the path traversed by the acid-base balance of the juice after its displacement

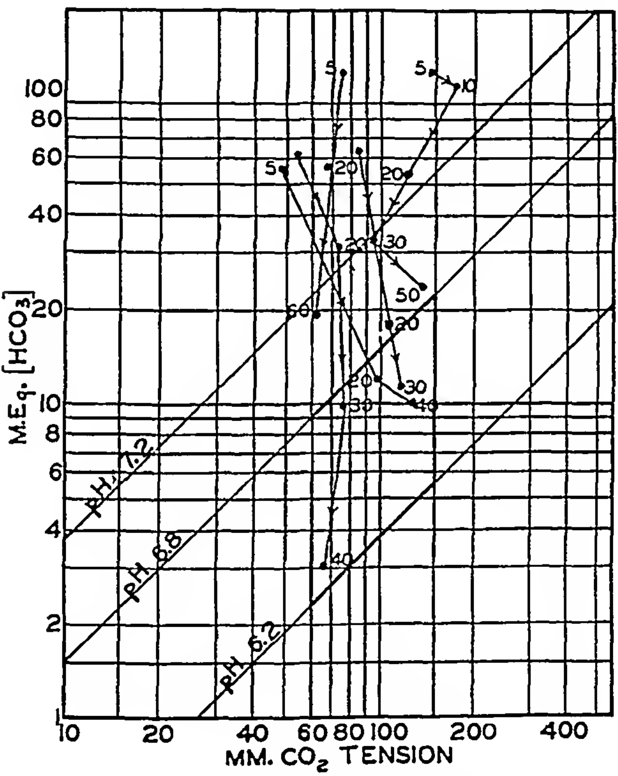

Fig. 6. Changes in acid-base balance following instillation of $NaHCO_3$ solution into the jejunum. The figures denote the time in minutes after $NaHCO_3$ administration that the specimen was obtained.

from its normal value by HCl. During the return to the normal area, the CO_2 tension remains remarkably constant.

Effect of $NaHCO_3$—The effect of the instillation of 100 cc. of solutions of $NaHCO_3$, varying in concentration from 0.15 to 0.6 M, is shown in Fig. 6. Serial samples were withdrawn at intervals

varying from 5 to 60 minutes following the alkali administration. The effect of the $NaHCO_3$ was to raise the HCO_3 and the pH of the juice. The CO_2 tension remained within normal limits.

The path taken by the acid-base balance in its return toward the normal area was opposite in direction to that taken after

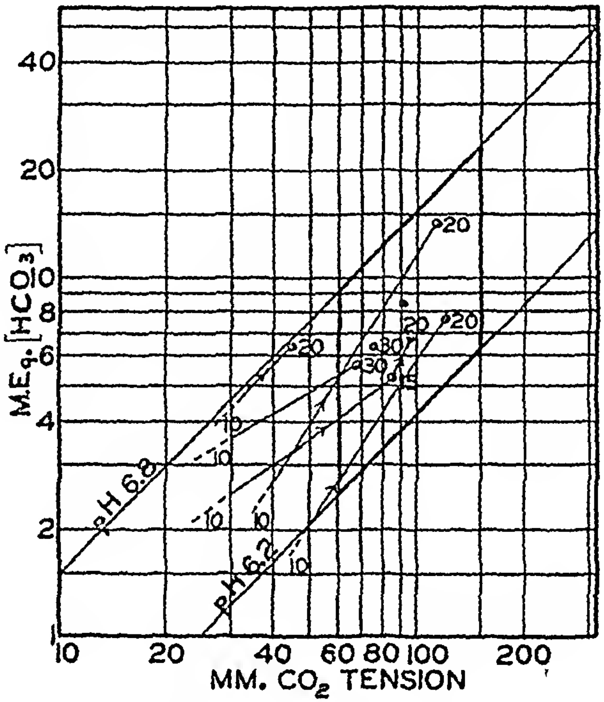

FIG. 7. Changes in acid-base balance following the instillation of 100 cc. of water into the jejunum. The figures denote the elapsed time in minutes since the water was administered.

displacement with HCl, and reached essentially normal values within 1 hour. Contrary to the results found after acid administration, no evidence of overcompensation was found after alkali administration.

Effect of H_2O—The effect of the instillation of 100 cc. of water on the acid-base balance of the jejunal juice was studied in five subjects (Fig. 7). The displacement produced was quite different from that observed in the previously described experiments. There was a proportional decrease in HCO_3 and CO_2 tension, leaving the pH essentially unchanged. Such changes could be

accounted for simply by the dilution of the juice without alteration in the concentrations of the acidic and basic constituents.

The return of the acid-base balance to normal followed paths consistent with the explanation that water was absorbed in order to restore osmotic equilibrium.

SUMMARY

1. Normal human jejunal juice tends to have a characteristic acid-base balance, with a bicarbonate concentration about one-third that of blood serum (8 mm. per liter), and a CO_2 tension about double that of venous blood (100 mm.), resulting in a slightly acid pH (6.5). These findings are consistent with those previously reported by Robinson (15) and by Herrin (16) for the juice from jejunal loops of dogs.

2. Displacement of the normal acid-base balance by acid, alkaline salts, or dilution results in a prompt return of the acid-base balance of the juice to normal values. It may be inferred, therefore, that regulation of the composition of the juice is to be regarded as a physiological phenomenon, characteristic of the cells responsible for the formation of the juice.

3. The high CO_2 tension at which the juice is maintained, in the absence of, or with the exclusion of gastric juice, suggests that the jejunal juice may originate from two sources, one of which produces an acid secretion and the other an alkaline secretion. The significance of these observations for the maintenance of the normal activity of intestinal enzymes merits further investigation.

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IODINE METABOLISM OF THE THYROID GLAND*

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Because of the similarity in structure between diiodotyrosine and thyroxine, Harington and Barger (1) suggested that the former is the precursor of thyroxine in the thyroid gland. The formation of thyroxine from diiodotyrosine has been demonstrated lately *in vitro* (2, 3). Recent data (4, 5) obtained with radioiodine used without carrier indicate the rates of incorporation of iodine into the various thyroid fractions but do not indicate the sequence of formation nor the respective sources of the finished components.

In a series of preliminary experiments with subphysiological doses of radioiodine, I^{130} (without carrier), we have attempted to determine whether diiodotyrosine is a natural precursor of thyroxine, whether inorganic iodine is normally present in the normal non-iodized thyroid (6, 7), and whether this inorganic iodine in the thyroid gland is the source of the iodine in the diiodotyrosine molecule.

Radioiodine (I^{130}) injected in subphysiological amounts as iodide and without carrier should behave like iodide already present in physiological amounts in the blood stream and thus is actually representative of the circulating iodide. The radioactivity found in the thyroid gland iodine fractions is the result of absorption and subsequent reactions in the gland which utilize newly arrived radioiodine obtained from circulating iodide.

The iodine fraction which is first formed or appears soon after

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the administration of the radioiodine should have the highest proportion of radioactivity. Later on, the proportion of radioactivity should rise in the other fractions as they are synthesized.

By the proportion of radioactivity we mean the percentage of administered radioiodine per microgram of I^{127}, which is called the specific radioactivity. Measuring the radioactive isotope of I^{130} and determining the chemical iodine (stable isotope I^{127}) from $\frac{1}{2}$ to 48 hours after injection of I^{130} without carrier, we can arrive at the specific radioactivities which are indicative of the normal relative rates of turnover of inorganic iodine, diiodotyrosine, and thyroxine in the thyroid gland. It is assumed in this type of experiment that the total combined iodine values (inorganic and organic) for both I^{127} and I^{130} remain constant for a particular animal under normal conditions, since this level should not be disturbed by the extremely small amount (subphysiological) of tracer radioactive iodine injected.

Method

Radioactive iodine was prepared by the proton bombardment of tellurium in the cyclotron, resulting in a product in which the isotope with a half life of 12.6 hours (I^{130}) was predominant. Radioactive iodine without carrier was separated from tellurium by oxidation with a chromic-sulfuric acid mixture, reduction with phosphorous acid, and distillation, according to Matthews, Curtis, and Brode's modification (8) of the Leipert procedure (9) for microdetermination of iodine. The radioactive iodine was collected in an alkaline medium, evaporated to a suitable volume, and partially neutralized with phosphoric acid to pH 7.5 and 8.0. The radioactive iodine was thus obtained as iodide presumably suitable for intravenous injection.

Six adult dogs, weighing between 20 and 40 kilos, received an injection of 10 cc. of radioactive iodine solution (containing practically no I^{127}) into the saphenous vein of the hind leg. At intervals of $\frac{1}{2}$, 8, and 48 hours after the injection, the animals were sacrificed by illuminating gas. The thyroid glands were trimmed, cut into small pieces, and dried in the chamber of a Fisher-Abderhalden drier at a temperature of 79° first for 1 hour, then removed and homogenized, and returned to the chamber which is then connected to a Hyvac pump until a constant weight of tissue was obtained.

Aliquots were then taken for the total inorganic and thyroxine iodine determinations (both I^{130} and I^{127}). Inorganic iodine was separated by the water extraction method according to Gutman *et al.* (10). As an added precaution against the possibility of extracting small amounts of protein containing iodine, trichloroacetic acid was added to the solution obtained after centrifugation. Traces of precipitate were obtained which contained insignificant amounts of radioactivity. The solution was then ashed and distilled according to the method of Matthews, Curtis, and Brode (8). The distillate obtained was evaporated and made up to volumes of 10 or 25 cc., depending upon the radioactivity content of the sample. 2 cc. of this volume were taken for radioactivity determinations by means of the dipping Geiger-Müller counter according to the method of Bale *et al.* (11). The remainder of the solution was then analyzed as usual for the determination of I^{127} (8).

Thyroxine was separated by the butyl alcohol extraction procedure according to Blau (12). After reduced pressure distillation to remove the butyl alcohol, the residue obtained was ashed and treated in the same manner as was the inorganic iodine. Total iodine was determined by treating an aliquot of desiccated thyroid in the same manner as were the aliquots measured for inorganic iodine and thyroxine after their separation, ashing, etc. Harington (13) has demonstrated that diiodotyrosine and thyroxine account for all of the organic iodine. Therefore, the iodine remaining after the *inorganic iodine* and *thyroxine* iodine were subtracted from the total iodine was considered the iodine of diiodotyrosine and is so designated in the rest of this paper. The determinations were made in duplicate and the iodine determinations were performed on the residue from the two extractions.

DISCUSSION

The results (Table I) indicate a general agreement with the values of I^{130} reported by Perlman *et al.* (4) for the sheep. The total quantity of radioactive iodine fixed in the gland rose throughout the three periods of observation to the highest level at 48 hours.

In order to study the rates of formation or turnover of inorganic and organic iodine it is necessary to compare the values for I^{130} with the values for I^{127} for each of the compounds studied. The

per cent of injected I^{130} divided by the amount of I^{127} present in the same iodine fraction gives a ratio which is called the "specific radioactivity" shown in Table I. This permits the comparison of the rate of formation of the different fractions in the same animal but does not permit direct comparisons with similar ratios obtained in other normal animals.

Table I

Rates of Turnover of Inorganic Iodine, Diiodotyrosine, and Thyroxine in Thyroid Gland

All determinations were made in duplicate. The figures in bold-faced type represent the relative specific radioactivities.

Time	No. of dogs	Total			Iodide			Diiodotyrosine			Thyroxine		
		Radioactive iodine*	Chemical iodine†	Specific radioactivity × 100	Radioactive iodine*	Chemical iodine†	Specific radioactivity × 100	Radioactive iodine*	Chemical iodine†	Specific radioactivity × 100	Radioactive iodine*	Chemical iodine†	Specific radioactivity × 100
hrs.		*per cent*	γ		*per cent*	γ		*per cent*	γ		*per cent*	γ	
½	15	8.2	863.0	0.96	0.27	43.7	0.62	7.6	652.7	1.17	0.30	166.6	0.18
				1.00			**0.66**			**1.27**			**0.19**
	14	15.2	420.5	3.56	3.30	54.0	0.61	11.3	265.0	5.30	0.40	96.0	0.47
				1.00			**0.17**			**1.46**			**0.13**
8	10	12.2	1966.0	0.62	0.82	165.0	0.50	10.3	1158.0	0.89	1.00	643.0	0.18
				1.00			**0.80**			**1.43**			**0.29**
	11	17.6	1366.0	1.29	1.30	145.0	0.87	15.3	807.0	1.90	1.00	364.0	0.24
				1.00			**0.68**			**1.49**			**0.19**
48	12	47.0	1637.0	2.87	1.50	232.0	0.69	43.2	1299.0	3.31	2.40	106.0	2.59
				1.00			**0.24**			**1.15**			**0.90**
	9	21.9	676.0	3.19							2.20	65.6	3.42
				1.00									**1.07**

* Per cent of total dose.

† Stable isotope I^{127}.

Since the proportion of radioactivity in the total iodine fraction is not constant from animal to animal, supposedly due to variations in blood iodide (I^{127}) concentrations and in the thyroid gland iodine concentrations of the different fractions among the animals, the specific radioactivity of the total thyroid iodine is made equal to unity in every experiment, and by proportion, the specific radioactivity of the iodine fractions in each individual thyroid is

adjusted accordingly. This new value is designated as the "relative specific radioactivity" and direct comparison may now be made among the values obtained in the case of one animal or animals with others, and permits graphing of the values. The "relative specific radioactivity" is recorded below the "specific radioactivity" in Table I. The "relative specific radioactivities" have been plotted in Fig. 1.

If what we titrate as *inorganic iodine* was produced during the extraction procedure by splitting off from thyroxine or diiodotyrosine or both, the specific radioactivity of the iodide at any time interval should be equal to either one, or lie somewhere in between the specific radioactivities of thyroxine and diiodotyrosine.

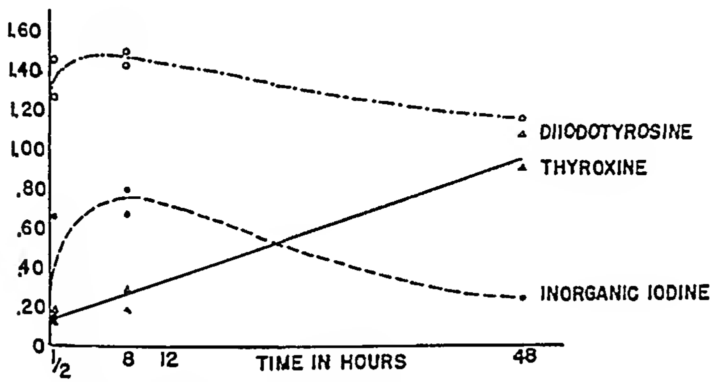

FIG. 1. Relative specific radioactivities in the thyroid gland

However, at 48 hours, the specific radioactivity of inorganic iodide is considerably below that of either thyroxine or diiodotyrosine. Likewise, any losses of I^{127} in the chemical extraction would be accompanied by proportional losses of I^{130}, thus leaving the ratios or specific radioactivity values undisturbed. Therefore, *thyroid inorganic iodine* is a true entity even in non-iodized glands (6, 7).

Inspection of Table I also shows that in the dogs sacrificed 30 minutes after the administration of radioactive iodide, the relative specific radioactivity of diiodotyrosine is much greater than that of inorganic iodide.

Since the radioactive iodine is administered intravenously in the form of iodide, and very soon penetrates into the extracellular fluid of the thyroid gland, a situation must exist soon after ad-

ministration wherein the specific radioactivity of iodide in the extracellular fluid of the thyroid gland is very high in relation to the specific radioactivity of the iodide inside of the thyroid cell. Since we measure the sum of the iodide contained in both the extracellular space and the thyroid cells, a high value in the relative specific radioactivity of iodide in the extracellular space might well be hidden. If we assume that all of the diiodotyrosine containing I^{130} has been newly manufactured (no exchange) (14, 4), then the findings of the high values at $\frac{1}{2}$ hour for diiodotyrosine (3 times that of iodide) indicates that the recently arrived inorganic I^{130} in the thyroid cell apparently could not have contributed in any great part to the iodine (I^{130}) which is part of the diiodotyrosine molecule, for if it did the specific radioactivity of the iodide should be higher or at least as high as that in the diiodotyrosine fraction.

Another possibility arises. Previous to $\frac{1}{2}$ hour, the specific radioactivity of iodide might have been very high and was decreasing at $\frac{1}{2}$ hour, and more subsequently. Inspection of Fig. 1 shows that this possibility is incompatible with the findings, for the relative specific radioactivity of iodide is increasing between $\frac{1}{2}$ and 8 hours. It is rather inconceivable that the relative specific radioactivity of iodide was very high previous to $\frac{1}{2}$ hour, then fell rapidly, and then rose again. It is also possible that some of the iodide present in the thyroid gland may have its origin from the breakdown of the newly formed diiodotyrosine molecule. Therefore most of the inorganic iodide coming from the interstitial fluid, and part of which may be destined to enter the thyroid cell as iodide, must be transformed immediately into diiodotyrosine before it is incorporated into the cells. Most likely this transformation takes place at the level of the cell membrane. Two other alternative explanations may be examined: (1) The reaction might take place in the extracellular fluid of the thyroid. This is unlikely because of the lack of specificity of extracellular fluids in general. (2) Diiodotyrosine might come to the thyroid already made by some other tissue or organs. This seems highly improbable in view of previous results indicating that diiodotyrosine as such does not enter the thyroid gland (14).

The fact that the relative specific radioactivities of diiodotyrosine in the animals sacrificed in $\frac{1}{2}$ hour are many times greater

than those found for thyroxine indicates that diiodotyrosine synthesis precedes thyroxine synthesis but does not necessarily mean that diiodotyrosine is a thyroxine precursor. That diiodotyrosine is a thyroxine precursor is demonstrated, however, by the additional evidence that there is practically a linear increase in the relative specific radioactivity of thyroxine throughout the period of time studied and to a magnitude which finally is relatively high. This means that the thyroxine originated from a compound having a high relative specific activity which has been maintained practically constant during this time. Inspection of Table I and Fig. 1 indicates that the relative specific radioactivity values of diiodotyrosine fulfil this requirement although the possibility that a small fraction of the total thyroxine formed may arise in another manner is not excluded.

From these data it is possible also to obtain some information concerning the absolute rate of formation or turnover of thyroxine. Hevesy and Hahn (15) have pointed out that the rate of formation of a substance can only be measured by the ratio of its specific radioactivity to the specific radioactivity of its precursor, and that the specific radioactivity of the latter must be kept constant. This ratio times 100 is equal to the per cent of the substance formed. An additional condition must be fulfilled, however, in order that the value be valid; namely, an insignificant amount, or none, of the radioactive compound must be removed. Since the relative specific radioactivity of diiodotyrosine apparently has remained more or less constant for most of the 48 hour period, and since not much "radioactive" thyroxine has been removed from the thyroid (otherwise the thyroxine curve would have a curvature which would be concave facing the abscissa), an approximate value for the rate of formation of thyroxine can be obtained. Using an average relative specific radioactivity of 1.3 for diiodotyrosine, we estimate that 1.55 per cent of the thyroxine is formed per hour.

SUMMARY

1. Inorganic iodine does exist as such in the normal, non-iodized thyroid gland of the dog.

2. The level of the relative specific radioactivity of the inorganic iodide in the thyroid gland does not rise high enough at any time

during the period of observations ($\frac{1}{2}$ to 48 hours) to indicate that it is the major source for the iodine of diiodotyrosine. Therefore, the conversion of the injected radioactive iodide into diiodotyrosine must take place at the level of the cell membrane. The results do not permit us to eliminate the improbable (14) hypothesis that diiodotyrosine arises elsewhere in the body and is secondarily fixed as such in the thyroid cell.

3. The diiodotyrosine fraction (obtained by subtracting the inorganic iodine and the thyroxine iodine from the total iodine) appears to be the natural precursor of thyroxine.

4. In the six dogs studied, it is estimated that 1.55 per cent of the thyroxine contained in the thyroid gland is formed per hour.

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A MICROBIOLOGICAL ASSAY FOR BIOTIN*

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Because of its rôle in the nutrition of animals and lower forms of life, biotin has become an important member of the B vitamins. Although widely distributed in nature, it occurs in exceedingly low concentration. To study its occurrence, properties, and functions, suitable methods for its determination are imperative.

Kögl and Tönnis (1) utilized yeast growth as a quantitative measure of the biotin in their preparations. Later Snell, Eakin, and Williams (2) reported a 16 hour assay for biotin which also utilized the response of yeast to added increments of biotin as a measure of the biotin present. West and Wilson (3) used a strain of *Rhizobium trifolii* for the determination of biotin. In all of these methods, growth is measured turbidimetrically.

In this paper is described a method for the assay of biotin which offers certain advantages over previously published methods. It is based on the fact that, under proper conditions, the titratable acidity produced by *Lactobacillus casei* ϵ is a function of the quantity of biotin present in the medium. Colored and turbid solutions, which give anomalous results with turbidity methods, can be assayed satisfactorily by this method. The same organism that is used in the riboflavin (4) and pantothenic acid (5) assays is used in this assay; thus no separate stock cultures are necessary.

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Culture and Inoculum

The stock cultures of *Lactobacillus casei* ε are prepared and maintained as described in the riboflavin method of Snell and Strong (4).

The inoculum for the assay is started 2 days before the assay is to be set up. A transfer is made from a stab culture to a tube of liquid medium (10 ml.) containing 0.5 per cent Difco Bacto-yeast extract,[1] 0.6 per cent sodium acetate, 0.5 per cent glucose, and 0.05 ml. of mineral salt Solutions A and B per tube. The culture is incubated at 37° for 24 hours and then transferred to the sterile biotin-free basal medium described below. A 2 per cent inoculum is used for the transfer; *e.g.*, 0.2 ml. per 10 ml. of the biotin-free basal medium. This inoculum supplies sufficient biotin for a good growth of the organism. This culture is incubated for 15 to 20 hours at 37°. 1 drop (0.05 ml.) of this suspension is used to inoculate each tube in the assay series.

Medium

The composition of the biotin-free basal medium is given in Table I.

Stock solutions of peroxide-treated hydrolyzed casein, peroxide-treated norit yeast filtrate, vitamins, and adenine, guanine, and cystine are prepared and kept in a refrigerator under toluene.

It is convenient to prepare a large quantity of the biotin-free basal medium complete except for glucose, sodium acetate, and mineral salts in advance. 1000 ml. (enough for 200 tubes) of this stock solution mixture are prepared from the stock solutions and solid compounds given in Table II.

The asparagine (C.P.) and tryptophane are dissolved in a small quantity of hot water and added to the mixture of the four stock solutions listed above. This solution is preserved with toluene and chloroform and kept in a refrigerator. It is important, obviously, that it be kept free from growth of contaminating microorganisms.

Peroxide-Treated Hydrolyzed Casein—100 gm. of Labco vitamin-free casein with 375 ml. of water and 125 ml. of concentrated

[1] The Difco Bacto-yeast extract, Difco Bacto-peptone, and Difco trypsinized liver were all obtained from the Difco Laboratories, Inc., Detroit.

H_2SO_4 are hydrolyzed for 15 hours at 120° in an autoclave. The 15 hour treatment hydrolyzes the casein sufficiently for the organism to utilize the hydrolysate, but it is not long enough to destroy completely the "eluate factor" required by *Lactobacillus*

TABLE I

Basal Medium

	per cent
Peroxide-treated hydrolyzed casein	0.50
" norit yeast filtrate (approximately)	0.15
Tryptophane	0.0075
Cystine	0.01
Asparagine	0.02
Glucose	1.00
Sodium acetate	1.00
	mg. per cent
Pyridoxine	0.02
Pantothenic acid	0.02
Riboflavin	0.01
Nicotinic acid	0.10
p-Aminobenzoic acid	0.01
Adenine	1.00
Guanine	1.00
Mineral salts, 0.5 ml. each of Solutions A and B in 100 ml. of medium	

TABLE II

Stock Solution Mixture

	ml.
Peroxide-treated hydrolyzed casein solution	200
" norit yeast filtrate solution	200
Vitamin solution	4
Adenine-guanine-cystine solution	200
	mg.
Tryptophane	150
Asparagine	400
Distilled water to make a total volume of 1000 ml.	

casei ϵ (6, 7). 640 gm. of $Ba(OH)_2 \cdot 8H_2O$, suspended in 400 ml. of boiling water, are added to the hydrolysate and the precipitated $BaSO_4$ is filtered off with Filter Cel. The filter cake is removed and thoroughly broken up in 500 ml. of hot water. The washings from the filter cake are added to the main filtrate. The pH of

the filtrate is adjusted to 3.0 with NaOH. The solution is then diluted to 1500 ml. with water and 15 ml. of superoxol[2] are added. The solution is thoroughly stirred and allowed to stand for 24 hours at room temperature (25–32°). The pH is then adjusted to 7.0 with NaOH and 1 per cent of powdered MnO_2 is added. The solution is mechanically stirred until no more oxygen is evolved (about 15 minutes), and filtered with suction. The filtrate is then diluted to 2000 ml. with water.

Peroxide-Treated Norit Yeast Filtrate—20 gm. of Difco Bacto-yeast extract are dissolved in 1 liter of distilled water and the pH is adjusted to 2.0 with concentrated H_2SO_4. 4 gm. of norit A are added, and the adsorption is carried out at 55–60° with mechanical stirring for half an hour. The norit is removed by filtration with the aid of Filter Cel. The pH of the filtrate is adjusted to 3.0 with 10 N NaOH and the volume is made up to 1300 ml. with water. 13 ml. of superoxol are added, and the solution is allowed to stand at room temperature for 24 hours. The pH is then adjusted to 7.0 with 10 N NaOH, and 13 gm. of powdered MnO_2 are added. The solution is mechanically stirred until the evolution of oxygen ceases. The MnO_2 is removed by suction filtration. The final volume should be about 1325 ml.

Vitamin Solution—200 ml. of a stock solution containing 100 γ of pyridoxine, 100 γ of calcium pantothenate, 50 γ of riboflavin, 500 γ of nicotinic acid, and 50 γ of *p*-aminobenzoic acid per ml. are prepared. This solution is stored with added toluene in a refrigerator.

Adenine-Guanine-Cystine Solution—1 liter of a stock solution containing 500 γ per ml. of adenine, 500 γ per ml. of guanine, and 1.0 mg. per ml. of cystine is prepared by dissolving these compounds in water with the addition of the least possible amount of H_2SO_4 to effect solution.

Mineral Salt Solutions—Solution A contains 25 gm. of K_2HPO_4 and 25 gm. of KH_2PO_4 dissolved in 250 ml. of water. Solution B contains 10 gm. of $MgSO_4 \cdot 7H_2O$, 0.5 gm. of NaCl, 0.5 gm. of $FeSO_4 \cdot 7H_2O$, and 0.5 gm. of $MnSO_4 \cdot 4H_2O$ dissolved in 250 ml. of water.

[2] Superoxol, 30 per cent H_2O_2, Merck and Company, Inc., Rahway, New Jersey.

Procedure

5 ml. per tube of the stock solution mixture, prepared as described above, are used. The correct amount of this solution, depending on the number of tubes in the assay, is pipetted out and placed in a mixing cylinder. 0.1 gm. per tube each of anhydrous glucose (C.P.) and anhydrous sodium acetate and 0.05 ml. per tube of each of the mineral Solutions A and B are added, and the solution is thoroughly mixed. The pH of the complete biotin-free basal medium is then adjusted to 5.6. 5 ml. of tbis solution are then added to each assay tube. Solutions to be assayed and the biotin for the standard curve are next added to the tubes. The final volume in each tube is made up to 10 ml. with distilled water.

It has been found desirable to use 19 × 150 mm. Board of Health tubes. The larger diameter of these tubes eliminates "blowing" and "wetting" of the plugs.

The tubes are plugged with cotton and autoclaved for 15 minutes at 120°. After cooling, the tubes are inoculated with 1 drop of the inoculum prepared as described above.

After inoculation the tubes are incubated for 3 days at 37°. After incubation the contents of each tube are titrated with 0.1 N NaOH. Brom-thymol blue may be used as an indicator in this titration, but phenol red gives a better end-point.

Standard Curve and Recoveries

The biotin used in this assay for the standard curve was very kindly supplied us by Dr. Vincent du Vigneaud. We have been able to demonstrate on different samples of biotin-free acid and biotin methyl ester from various sources that the organism, *Lactobacillus casei* ϵ, is unable to utilize the methyl ester. By hydrolyzing the methyl ester samples as described by du Vigneaud *et al.* (8) the full activity of the biotin was restored. Consequently, the biotin to be used as a standard for this assay should be in the form of the free acid.

The range of biotin used for the calibration curve is from 40 to 1000 micromicrograms per tube. A curve similar to the one illustrated in Fig. 1 is obtained by plotting the micromicrograms of biotin as abscissa and the ml. of 0.1 N NaOH used in titration

as ordinate. The extremities of the curve as a rule will give anomalous results; therefore, the samples to be run should be diluted so that their titers do not come at these extremities.

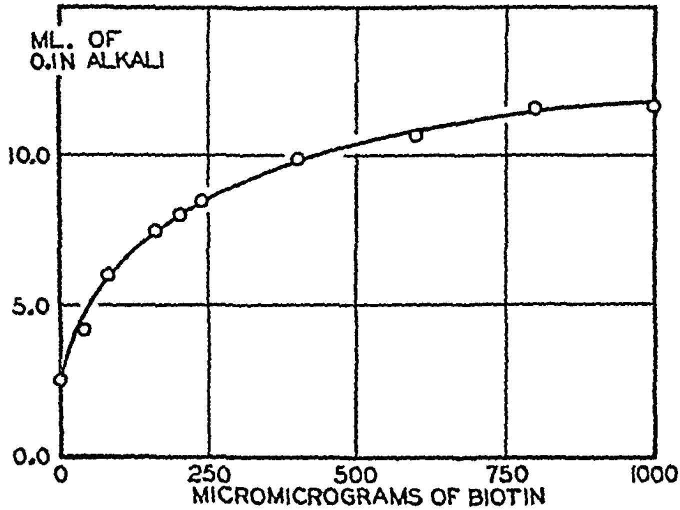

FIG. 1. Standard curve for assay

TABLE III
Recovery of Added Biotin

Material	Dilution	Sample of final dilution	Biotin in sample	Biotin added	Calculated	Value obtained	Recovery
		ml. per tube	*micromicrograms*	*micromicrograms*	*micromicrograms*	*micromicrograms*	*per cent*
Human urine	1:250 ml.	2.5	160	80	240	255	106
		2.5	160	120	280	305	108
		2.5	160	160	320	330	103
Pork liver (dried)	0.72 gm. per 250 ml.; 1 to 100 ml.	1.0	60	80	140	145	103
		1.0	60	120	180	192	106
		1.0	60	160	220	230	103
Egg yolk (dried)	1 gm. per 1000 ml.; 1 to 10 ml.	0.5	42	80	120	122	101
		0.5	42	160	202	200	100

In Table III are listed the recoveries of biotin added to different materials. As can be seen in the last column, the recoveries are all within 10 per cent.

Biotin Content of Biological Materials

Snell, Eakin, and Williams (2) showed that biotin occurred in liver in a combined form from which it was released by hydrolysis. Lampen, Bahler, and Peterson (9) in a recent paper have demonstrated that biotin occurs in a combined form in many materials. The optimum conditions of acid hydrolysis have also been studied by the latter group.

With the exception of yeast extract, Curbay BG, Galen "B," urine, and peptone, all of the materials listed in Table IV were

TABLE IV

Biotin Content of Some Biological Materials

The results are given in millimicrograms per gm. of dry weight, except where indicated otherwise.

Sample	Assay 1	Assay 2	Assay 3	Mean
Yeast extract	1028	1140	1081	1081
Trypsinized liver	1850	1663	1898	1804
Curbay BG	2050	2228	2122	2133
Galen "B"	373	364	392	376
Human urine*	31.8	31.2	30.6	31.2
Milk*	12.7	11.4	13.3	12.5
Beef blood*	0.965	0.914	0.755	0.878
Egg yolk	681	681	757	706
Malt sprouts	153	153	153	153
Corn	78.3	77.7	78.3	78.1
Lean pork	69.2	59.0	52.8	60.3
Tomato	432	425	432	430
Carrot	167	164	152	161
Peptone	169	146	154	156

* Per ml.

hydrolyzed in 3 N H_2SO_4 for 1 hour at 120° in an autoclave. The H_2SO_4 was removed with $Ba(OH)_2$, the $BaSO_4$ was washed thoroughly with water, and the volume of the filtrate adjusted in a volumetric flask. The materials not treated in this manner were either liquids or water-soluble products. The trypsinized liver[1] and Curbay BG[3] were autoclaved for 1 hour with 50 volumes of water at 120°. The yeast extract, Galen "B,"[4] and peptone[1]

[3] Fermentation residue from the United States Industrial Alcohol Company, New York.

[4] Vitamin B complex from the Galen Company, Inc., Berkeley.

were merely taken into solution, and the appropriate dilutions were made from this solution. The urine was diluted directly and assayed.

While previously published microbiological methods have usually included a table of vitamin contents of various foods and natural products, the number of times the assay was repeated on a given sample has generally not been stated. In order to ascertain the accuracy of our assay and also to arrive at a more nearly correct value for the biotin content of the sample assayed, we have included each sample in three separate assays. From the values obtained in this manner for each sample, the mean value was calculated.

SUMMARY

A microbiological assay for biotin in which *Lactobacillus casei* ε is utilized has been described.

The assay differs from other biotin assays in that the amount of biotin is calculated from the titratable acidity produced. Colored and turbid solutions can be assayed satisfactorily by this method.

Recoveries of biotin added to different natural products indicate that the method has an accuracy of ±10 per cent.

The biotin content of some biological materials is given. Each of these samples was assayed three times at different intervals.

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THE ESTIMATION OF SERUM INORGANIC PHOSPHATE AND "ACID" AND "ALKALINE" PHOSPHATASE ACTIVITY*

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In 1930, Kay (6, 7) introduced a method for the quantitative determination of "alkaline" phosphatase activity of blood, employing sodium β-glycerophosphate as the substrate. Of the many modifications which have been introduced in which this substrate is employed, Bodansky's (3, 4) method seems to have found the greatest usage because of its short incubation period and its accuracy. For our studies certain fundamental changes in the technique for the quantitative estimation of serum phosphatase activity and inorganic phosphate were necessary. Presented in this communication are procedures for the simultaneous determination of serum inorganic phosphate and "acid" and "alkaline" phosphatase activity. The estimation of all three tests may be made on 0.40 cc. of serum in the macrotechnique, 0.06 cc. in the microtechnique. Only one photometric calibration curve or one set of phosphorus standards for visual colorimetry is required.

The unit of phosphatase activity is defined here as follows: Each unit of serum phosphatase activity is equivalent to 1 mg. of phosphorus as phosphate ion liberated during 1 hour of actual incubation at 37° with a substrate containing sodium β-glycerophosphate, hydrolysis not exceeding 10 per cent of the substrate; and at

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† The material on the microtechnique contained in this paper is part of a thesis submitted by Miss Lois M. Jones in partial fulfilment of the requirements for the degree of Master of Arts.

optimum pH of the reaction mixture, for "acid," 5.00 ± 0.15 and for "alkaline," 9.30 ± 0.15. This definition is similar to that of Levene and Dillon (10) and that of Bodansky (4) for "alkaline" activity. However, it differs in the elimination from its calculation of mathematical corrections for the interfering substances in the phosphorus determination and for variances in the incubation period, in its application to "acid" phosphatase, and in its estimation at optimum pH.

Reagents

The reagents for the determination of inorganic phosphate are those of Kuttner, Cohen, and Lichtenstein (8, 9) with suggestions by Raymond and Levene (11) and Bodansky (2). That these must be most carefully prepared cannot be overemphasized. The molybdic acid reagent is made up daily by adding 1 part of 7.5 per cent sodium molybdate to 1 part of *cold* 10 N sulfuric acid with constant shaking. The dilute stannous chloride solution, prepared by adding 0.2 cc. of the stock reagent (6 gm. of $SnCl_2 \cdot 2H_2O$ plus 10 cc. of concentrated HCl, kept in the refrigerator) to 100 cc. of cold distilled water, is used within 4 hours and is kept cold by means of an ice bath. Both of these solutions must be water-clear. For the "working" phosphate standard containing 2 γ of P per cc., dilute 2 cc. of the stock phosphate solution (0.4394 gm. of KH_2PO_4 in 1 liter of distilled water) to 100 cc.

The stock substrate is prepared as follows: Into a 500 cc. volumetric flask introduce successively 15 cc. of petroleum ether (b.p. 20–40°, J. T. Baker, Analyzed Special), about 400 cc. of water, 5.0 gm. of sodium β-glycerophosphate (C.P., Eastman, $C_3H_5(OH)_2$-$NaPO_3 \cdot 5\frac{1}{2}H_2O$, mol. wt. 315), and 4.24 gm. of sodium diethyl barbiturate (C.P., Merck, mol. wt. 206.1). Bring the aqueous level up to the 500 cc. mark with distilled water. Transfer to a glass-stoppered bottle containing about an inch layer of petroleum ether. This is kept in the refrigerator.

For the preparation of "working" substrate carefully pipette into a 100 cc. volumetric flask containing 5 cc. of petroleum ether 50 cc. of stock substrate. Add acid or alkali as indicated in Table I and bring the aqueous level up to the 100 cc. mark with

distilled water. *Check the pH.* These substrates are kept in the refrigerator.

Macrotechnique—Obtain 1 to 2 cc. of unhemolyzed serum. If postponement of the test is desirable, place the serum in the *freezing unit* of the refrigerator. No increase in activity is noted in serum stored in this manner; a less than 5 per cent decrease is obtained at the end of 7 days. (This is in contrast to Bodansky's (4) findings.)

Add 1 part of serum (0.1 cc., minimum) to 10 parts of the proper substrate warmed to 37° ("total" phosphate). Incubate at this temperature for exactly 1 hour. 9 parts of 10 per cent trichloroacetic acid are added to stop the enzyme action and to precipitate the proteins. At about the same time, to 1 part of serum (0.2 cc., minimum) add 9 parts of trichloroacetic acid (inorganic phosphate). Mix by inversion, and within 5 to 10 minutes after the addition of trichloroacetic acid centrifuge at about 2500 R.P.M. for 10 minutes. Decant the clear supernatant fluid into clean tubes.

TABLE I

Acid or Base Added to Substrate

Substrate	Volume of serum used (see Table II)	Acid or base	pH ±0.05 at 25°
Alkaline, A	1.0 Macrotechnique	2.8 cc. 0.1 N NaOH	10.9
" B	0.5 " 1.0 Microtechnique	1.5 " 0.1 " "	10.6
" C	0.5 "	1.0 " 0.1 " "	10.4
" D	0.1 Macrotechnique 0.1 Microtechnique	0.2 " 0.1 " "	9.8
Acid	Macro- and microtechnique	5.0 " 1.0 " HOAc	5.0

To 1 cc. of "inorganic" protein-free fluid in a 25 cc. Erlenmeyer flask are added 5 cc. of 0.10 N NaOH. To 1 cc. or less of "total" phosphate protein-free fluid is added sufficient 0.05 N NaOH to make the total volume 6 cc. Add 2 cc. of molybdic acid with shaking to each flask as well as to a "blank" flask containing 6 cc. of water. Next add 2 cc. of dilute stannous chloride and make readings 6 minutes later. Since the color development begins with the addition of the reducing reagent, it is added to each of the flasks at definitely measured time intervals.

Microtechnique—Where venipuncture is considered inadvisable, as in serial studies on infants and on small animals, capillary blood may be collected from a deep puncture wound by use of a capsule (Fig. 1). When a firm clot is formed, loosen it with a fine stylet wire. For centrifugation the capsule is packed with cotton in a tube the size of which depends upon the number of capsules and centrifuged for 10 minutes at 1500 R.P.M. With a Sahli hemoglobin pipette, 0.02 cc. of serum is immediately measured into each of two 12 × 75 mm. tubes (Kahn serological tubes) containing 0.2 cc. of water. The tests may be run at once or the tubes tightly corked and placed in a freezing tray.

One tube of diluted serum ("total" phosphate) is warmed to 37° in a water bath. To it is added 0.4 cc. of the proper substrate which has also been warmed to 37°. After 60 minutes, 0.4 cc. of

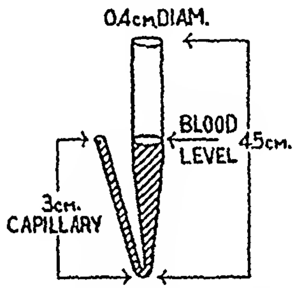

FIG. 1. Blood-collecting capsule

10 per cent trichloroacetic acid is added. To a second tube (inorganic phosphate) is added 0.4 cc. of distilled water instead of substrate. 0.4 cc. of 10 per cent trichloroacetic acid is added to this tube at the same time it is to the tube containing substrate. The tubes are centrifuged for 5 minutes at 2000 R.P.M. The resulting supernatant fluid, which should be water-clear, is decanted into another tube.

0.5 cc. of protein-free fluid is placed in a 15 × 85 mm. test-tube (Kolmer serological tube) which is broad and permits rapid mixing while the reagents are being added. 0.7 cc. of 0.10 N sodium hydroxide is added, followed by 0.4 cc. of molybdic acid reagent. The tube is shaken during this addition and tapped afterwards to insure washing down any drops adhering to the sides of the tube. At the same time a "blank" is prepared by adding 0.4 cc. of

molybdic acid reagent to 1.2 cc. of double distilled water. When it is necessary to employ lesser quantities of protein-free fluid, owing to excessive phosphate concentration, the final volume before the addition of molybdic acid is made up to 1.2 cc. with 0.05 N sodium hydroxide. At this point the solution in all the tubes should have a volume of 1.6 cc. and should be water-clear, with no trace of color. Then, 0.4 cc. of cold, dilute stannous chloride is added to each tube with the same precautions as in the addition of the molybdic acid. As in the macrotechnique, readings are made 6 minutes later.

Colorimetry—The density of the color developed by the reduction of phosphomolybdic acid can be read on a photometer[1] or a spectrophotometer. With the former, employ a filter with a maximum transmission at approximately 600 mμ. Construct a calibration curve by pipetting 0, 2, 4, and 6 cc. of the "working" phosphate standard solution. Make the total volume up to 6 cc. with distilled water. Add 2 cc. of molybdic acid, followed by 2 cc. of stannous chloride. These will give readings for 0, 4, 8, and 12 γ of P. If desired, more points on the curve may be determined. The macro curve is applicable to the micro phosphate technique. With the instrument employed in this investigation, the phosphorus concentration-galvanometer unit curve, plotted on semilogarithmic graph paper, is linear.

With a visual colorimeter, two standards containing 4 and 8 γ of P, as well as a "blank," are prepared. As in the photometric application, readings of the unknown are made 6 minutes after the addition of stannous chloride. Data from photometric studies on the relationship of time to color development show that satisfactory results can be obtained by comparison against 4 or 8 γ standards, the color development time of which is not less than 4 minutes nor more than 8 minutes. In other words, about eight unknown readings may be made with one set of standards. The standards described above for the macroprocedure are employed for the micro modification. With concentrations under 12 γ of P in the macroprocedure and 2.4 γ of P in the microprocedure, errors

[1] The Cenco-Sheard-Sanford photelometer was employed in this investigation. The orange filter is standard equipment. No special accessories were required for the microtechnique. Readings were made on a 2 cc. volume contained in a 10 cc. fused cell, 1 cm. thick.

due to deviation from Beer's law are slight when comparisons are made against the closest standard.

Selection of Amounts of Serum and Protein-Free Fluid—There are two limitations in the selection of the proper volume of serum or protein-free fluid to be employed in the test: First, the hydrolysis should not exceed 10 per cent of the substrate; second, the concentration of phosphate should not exceed 12 γ of P in the macro- or 2.4 γ of P in the microprocedure. The procedures described in detail above are applicable to serum having "acid" or "alkaline" phosphatase activity of less than 24 units or 48 units when half

TABLE II

Selection of Volumes of Serum and Protein-Free Fluid at Various "Acid" and "Alkaline" Phosphatase Activity Ranges; Calculation Factors

Range of phosphatase activity	Serum	Protein-free fluid	Calculation factors	
			Macrotechnique	Microtechnique
units	*volume*	*volume*		
0– 24	1.00*	1.00†	2.00	2.04
8– 48	1.00	0.50	4.00	4.08
16– 96	0.50	0.50	8.00	8.16
32–192	0.50	0.25	16.00	16.32
160–	0.10	0.25	80.00	81.60
Inorganic phosphate		1.00	1.00	2.04

* 0.50 cc. of serum to 5.0 cc. of substrate (macrotechnique); 0.02 cc. of serum to 0.4 cc. of substrate (microtechnique).

† 1.00 cc. of protein-free fluid (macrotechnique); 0.50 cc. of protein-free fluid (microtechnique).

volumes of protein-free fluid are used. For sera having higher activities the various volumes to be employed are tabulated in Table II. Volume correction for a decreased amount of serum is made with distilled water. Approximation of high "alkaline" phosphatase activity may be made by employing smaller quantities of protein-free fluid than are indicated in Table II. When the range is established, the proper substrate and volumes of sera and protein-free fluid are selected and the test repeated, if accurate results are desired. For "acid" phosphatase activity, one substrate suffices for varying volumes of serum.

Calculations

The calculation factors in Table II are derived as follows:

$$\text{Calculation factor} = \frac{100 \text{ cc. serum}}{A} \times \frac{10}{B} \times \frac{1}{1000}$$

where A is the serum equivalent (in cc.) in the actual volume of protein-free fluid used in the color development, and B is the final volume of the color solution. In the macrotechnique, the latter is 10 cc.; in the microtechnique, 2 cc. The 1/1000 is to convert micrograms to mg. Accordingly, the calculations for both macro- and microprocedures are greatly simplified.

Serum Inorganic and "Total" Phosphate—Photometry (micrograms of P from the macro calibration curve), micrograms of P $\times$ calculation factor = inorganic or "total" phosphate in mg. of P per 100 cc.; visual colorimetry (micrograms of P in the macro standard), $S/R \times$ micrograms of P $\times$ calculation factor = inorganic or "total" phosphate in mg. of P per 100 cc.

Serum "Acid" or "Alkaline" Phosphatase Activity—"Total" minus inorganic = units per 100 cc.

Influence of Interfering Substances in Estimation of Inorganic Phosphate—Bodansky (2) found that errors up to 11 per cent in the estimation of inorganic phosphate can be attributed to varying quantities of glycerophosphate plus trichloroacetic acid in the protein-free filtrate. Application of his correction factors reduced this error to less than 2 per cent in phosphate concentrations of 12 to 36 γ of P. It was found in the investigation reported here that 0.90 cc. of 10 per cent trichloroacetic acid and 0.45 cc. of 10 per cent trichloroacetic acid plus 0.50 cc. of glycerophosphate caused diminution in color intensity (range, 0.0 to 12 γ of P) of approximately 7 and 4 per cent respectively. With lesser quantities of trichloroacetic acid plus glycerophosphate, the effects were correspondingly smaller.

By reducing the acidity of the protein-free fluid in either the macro- or microtechnique with dilute alkali, *the effects of trichloroacetic acid and of trichloroacetic acid plus glycerophosphate in the diminution of color development were completely negated,* thereby eliminating the use of several calibration curves or correction

tables. One calibration curve suffices, then, for the determination of serum inorganic phosphate as well as the "total" (inorganic plus hydrolysate) phosphate in both the macro- and microtechniques. In solutions of known phosphate concentrations below

TABLE III

pH and Serum or Plasma "Alkaline" Phosphatase Activity Obtained with Sodium β-Glycerophosphate Substrate, As Determined by Various Investigators

Investigators	Material tested	Buffer	Temperature	Period of hydrolysis	pH of reaction mixture
			°C.	hrs.	
Kay (6)	Rat plasma	Glycine + NaOH	38	48	8.8–9.1 (Optimum)
	Human plasma				" "
	Rabbit plasma				" "
Roche (12)	Horse serum		38	19	9.0 (Optimum)
	Rabbit serum				
	Guinea pig serum				
Bodansky (4)	Human serum	Sodium diethyl barbiturate	37	½–1	8.6 (About)
Belfanti, Contardi, and Ercoli (1)	Rabbit serum	Sodium acetate + sodium diethyl barbiturate	37	16	9.7 (Optimum)
	Horse serum				9.5 (Optimum)
Woodard, Twombly, and Coley (14)	Human serum	HCl + sodium diethyl barbiturate	37	⅓–2	8.2* (Approximate)
		NaOH + sodium diethyl barbiturate			8.7* (Approximate)

* Alkaline phosphatase activity was determined at pH 8.6 by interpolation.

12 γ of P, the macrotechnique has an error of less than 1 per cent. In duplicate inorganic phosphate determinations on single *serum* specimens, the error is less than 3 per cent (on duplicate determinations on *protein-free fluid* the error is even less). With the microprocedure the actual error on serum is less than 4 per cent.

Optimum pH for Serum "Acid" and "Alkaline" Phosphatase Activity[2]—The pH of the reaction mixture in the determination of serum "alkaline" phosphatase activity, with sodium β-glycerophosphate substrate, as reported by various investigators is tabulated in Table III. In respect to serum "acid" phosphatase activity with this substrate, Gutman and Gutman (5) have presented conclusive evidence that the optimum pH is 5.0. Woodard and Higinbotham (13) determined serum "acid" phosphatase activity at pH 6.4 in an unbuffered sodium β-glycerophosphate substrate. In view of these slight but significant variances we have reinvestigated the serum phosphatase activity in both the

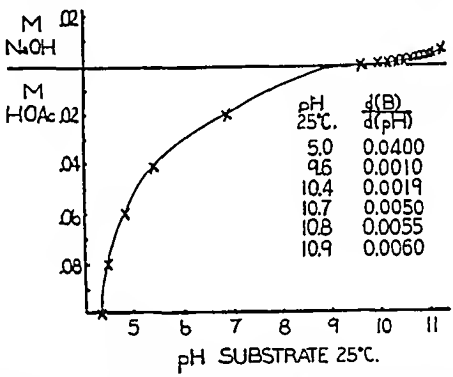

FIG. 2. Titration curve of substrate (0.0206 M sodium diethyl barbiturate and 0.0160 M sodium β-glycerophosphate); buffer capacities, $d(B)/d(pH)$.

"acid" and "alkaline" range, with particular reference to the latter.

In Fig. 2 the data obtained by the titration on sodium β-glycerophosphate + sodium diethyl barbiturate are presented. Examination of the curve demonstrates that the substrate is not a strong

[2] A glass electrode electrometer was employed for all pH determinations. The instrument was checked at different temperatures with Clark and Lubs buffer solutions at pH 4, 7, 9, and 10, and counterchecked against another electrometer. In control studies it was found that no change in pH was encountered during the 1 hour hydrolysis period. No hydrolysis was detected when the various substrates were incubated without the presence of serum; likewise there was no significant change in the inorganic phosphate when serum was incubated in buffer solution (without sodium β-glycerophosphate) at various pH.

buffer especially on the alkaline side. The observed changes in pH obtained by adding serum to substrate are shown in Fig. 3. The pH of the substrate alone was determined at 25°; of serum-

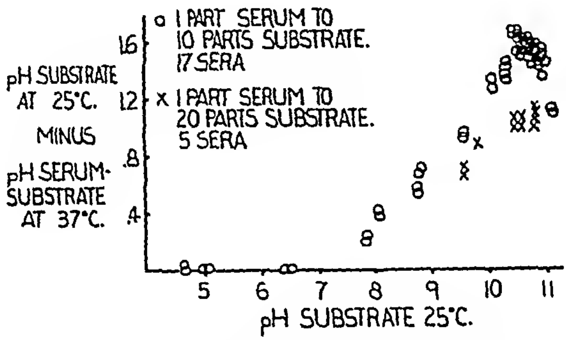

FIG. 3. Scatter diagram showing differences in the pH of the substrate at 25° and of the serum-substrate at 37°.

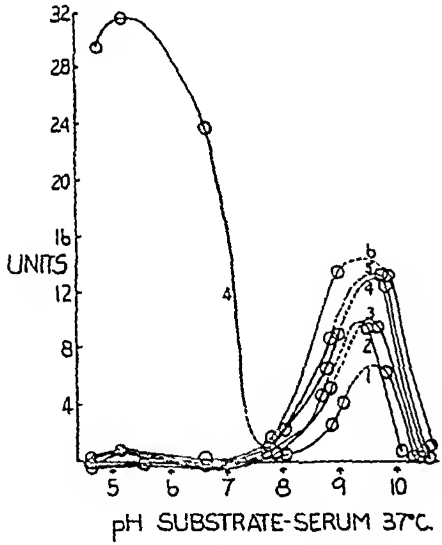

FIG. 4. pH-activity curves. Curve 1, normal human serum; Curves 2, 3, 5, and 6, sera from cases of obstructive jaundice; Curve 4, serum from a case of carcinoma of the prostate metastatic to the bone. 10 parts of substrate (0.0206 M sodium diethyl barbiturate and 0.0160 M sodium β-glycerophosphate) to 1 part of serum.

substrate, at 37°, the hydrolysis temperature for the estimation of serum phosphatase activity. It must be noted that approximately 30 per cent of the apparent lowering in pH in the higher

TABLE IV

Serum "Alkaline" Phosphatase Activity at pH 8.6 to pH 10.0

The values below are in mg. of P hydrolyzed per 100 cc. in 1 hour; 0.0160 M sodium β-glycerophosphate, 0.0206 M sodium diethyl barbiturate.

Serum No.	M NaOH in substrate											$\frac{(a)}{(c)}$	$\frac{(b)}{(c)}$
	0.0000	0.0004	0.0008	0.0012	0.0016	0.0020	0.0024	0.0028	0.0032	0.0036	0.0072		
	Substrate pH at 25°												
	9.61	10.08	10.36	10.51	10.65	10.74	10.80	10.88	10.95	11.00	11.15		
	Substrate-serum pH at 37°*												
	8.6 (a)	8.7	8.8	8.9	9.0 (b)	9.1	9.2	9.3 (c)	9.4	9.5	10.0		
1	2.2							3.4				0.65	
2	2.3							4.5				0.51	
3	2.7							4.7				0.57	
4	3.0				5.0			6.3				0.48	0.80
5	3.2							5.8				0.55	
6	3.5							5.5				0.63	
7	3.7							5.7				0.65	
8	3.9							6.9				0.56	
9	4.1							6.9				0.59	
10	4.1	4.8	5.6	6.7	7.1	7.9	7.9	7.9	7.9	7.3	2.1	0.52	0.90
11	4.4				6.7			8.4				0.52	0.80
12	4.6				7.4			9.0				0.51	0.82
13	4.9							7.5				0.65	
14	5.0							8.6				0.58	
15	6.6				11.0			12.6				0.52	0.88
16	7.0				11.0			13.8				0.51	0.80
17	8.4	9.6	11.0	12.0	13.0	13.8	14.4	14.0	13.8	13.0	3.4	0.60	0.93
18	9.0							14.2				0.63	
19	15.2							20.0				0.76†	
20	26.3							35.5				0.74†	
Range												0.48–0.65	0.80–0.93
Mean												0.565	0.85

* pH to the nearest 0.05; 1 part of serum to 10 parts of substrate.
† Not included in the mean.

alkaline range is due to the difference in temperature. The effect of serum on the pH of the substrate decreases with the pH; at

pH 5 the effect is negligible. When 1 part of either fresh or preserved serum is added to 10 parts of substrate at pH 10.8 to 10.9 at 25°, the pH at 37° of the final reaction mixture is between 9.2 and 9.4. The effect of adding 1 part of serum to 20 parts of substrate is appreciably less. Results obtained here point out the variations in pH of the final reaction mixture which can be observed due to even slight deviations in the pH of "alkaline" substrate or in the proportion of serum added.

It is evident from the six pH-activity curves in Fig. 4 that the serum "alkaline" phosphatase activity is highest in the pH range of 9.1 to 9.7, and that the slopes of the pH-activity curve on either side of this range are very steep. At approximately pH 7.5 and 10.5 there is very little "alkaline" phosphatase activity under the conditions of our experiments. The optimum pH for serum "acid" phosphatase activity as revealed by Curve 4 is about pH 5. This is in agreement with the work of Gutman and Gutman (5). A more detailed study in the pH range 8.6 to 10.0 of "alkaline" phosphatase activity is presented in Table IV. From the data on Sera 10 and 17 in Table IV and by interpolation of the curves in Fig. 4, it has been definitely shown that the optimum phosphatase activity is obtained when the substrate-serum reaction mixture has a pH of 9.3 ± 0.15 at 37°. Experiments on eighteen sera (see Table IV) show that 48 to 65 per cent of the optimum "alkaline" phosphatase activity is obtained at pH 8.6 in sera of less than 15 units; in sera with higher activity the differences at the optimum and pH 8.6 become less. At pH 8.6 a deviation of 0.1 pH unit will cause a change in the phosphatase activity of about 14 per cent; at pH 9.3, a similar deviation will cause a change of only 3 per cent.

Results

The actual analytical error by either the macro- or microtechnique for the estimation of phosphatase activity is less than 5 per cent in sera of normal or high activity. Normal values for "alkaline" phosphatase in adults range from 2.2 to 8.6 units. Results of our investigation of the serum "acid" phosphatase, the details of which will be presented elsewhere, indicated a range of 0.0 to 1.1 units per 100 cc. in twenty healthy adult subjects and in 140 control patients. In cases of proved carcinoma of the prostate

metastatic to the bone, "acid" phosphatase activity of 1.2 to 31.7 units was obtained.

SUMMARY

Micro and macro photometric techniques are described for serum inorganic phosphate and "acid" and "alkaline" phosphatase activity. Sodium β-glycerophosphate is the substrate used. On this basis the unit of phosphatase activity for both enzymes is comparable.

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SERUM INORGANIC PHOSPHATE AND "ALKALINE" PHOSPHATASE ACTIVITY IN HYPOPHYSECTOMIZED RATS*

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In 1932, Thompson and Collip (11) in a review of the influence of the endocrine glands on the calcium and inorganic phospbate content of blood serum made no mention of the pituitary. Since that time several observations suggest that it should be considered also. Schour and van Dyke (7) found significant defects of calcification of the teeth of hypophysectomized rats which were corrected more or less completely by replacement therapy. Pugsley and Anderson (6) found that the negative calcium balance of hypophysectomized rats maintained on a low calcium diet could be changed to a positive balance by the administration of anterior pituitary growth hormone.

Mortimer (5) presented a thorough radiological investigation of the effect of hypophysectomy and hormone administration upon the bones of the rat using the skull for particular study. Hetherington and Weil (3) employing the same experimental animal found that hypophysectomy resulted in a negative calcium balance, while phosphorus was reduced but maintained a positive balance. Snyder and Tweedy (10) in 1941, using both rats and guinea pigs, found a slight increase in inorganic phosphate with no change in calcium following daily injections of alkaline anterior pituitary preparations.

In 1935 a case of acromegaly in which there was an increased urinary excretion of calcium accompanied by marked osteoporosis was described by Scriver and Bryan (8). Bauer and Aub (1) (1941)

* A portion of the expenses of this project was defrayed by grants from the Comly Fund and the McNeil Laboratories.

in reporting calcium-phosphorus metabolism studies on five cases of acromegaly conclude that "the pituitary gland must be considered as one of the factors capable of altering calcium and phosphorus metabolism."

TABLE I

Serum "Alkaline" Phosphatase Activity and Inorganic Phosphate in Hypophysectomized and Control Rats

Date		Weight		Alkaline phosphatase		Inorganic phosphate	
		Range	Average	Range	Average	Range	Average
		gm.	*gm.*	*units*	*units*	*mg. per cent*	*mg. per cent*
Apr. 3*	C., H.†	140–155	150	20– 55	40	4.8–6.8	5.5
" 15	C.	160–180	170	49– 73	59	6.2–7.0	6.7
	H.	137–144	141	49– 85	72	4.2–5.8	5.0
" 21	C.	167–184	176	56– 76	68	6.8–8.7	7.4
	H.	135–142	138	55–144	113	4.4–5.2	4.6
" 28	C.	187–200	192	64– 94	75	6.8–8.7	7.6
	H.	130–140	134	85–154	109	3.8–4.4	4.2
May 6	C.	189–205	197	57– 98	78	6.2–6.8	6.3
	H.	125–140	130	60–130	109	3.6–4.4	4.0
" 13	C.	193–210	203	57– 81	77	6.8–8.3	7.6
	H.	122–142	129	82–181	114	5.2–6.2	5.6
" 20	C.	202–223	210	63– 89	74	5.8–7.4	6.4
	H.	122–142	132	80–163	123	4.0–8.0	5.7
" 27	C.	208–225	212				
	H.	120–141	129	79–194	128	5.0–6.4	5.4
June 16	C.	2 rats		49, 78	64	7.0, 8.3	7.6
	H.	3 "		110–130	122	4.0–4.8	4.4

* Hypophysectomy, April 5.

† C. represents a control group of six rats; H. a hypophysectomized group of eight rats.

Recently "alkaline" phosphatase activity of blood serum has come to be recognized as a valuable supplement to calcium and inorganic phosphate determinations. The present study was undertaken to follow the effect of hypophysectomy upon phosphatase activity and inorganic phosphate concentration in the blood of the white rat.

EXPERIMENTAL

The animals used were laboratory white female rats from three litters. The first litter contained six, three of which were hypophysectomized when 84 days old; the second contained five, three of which were operated on when 83 days old; the third litter contained three, two of which were hypophysectomized when 70 days old. This gave a total of eight hypophysectomized animals and six controls. Stock diet[1] and water were given *ad libitum* and approximately 20 cc. of milk fed each animal daily.

Records were kept of the weight changes for each animal. The control rats gained approximately 70 gm. each; the hypophysectomized animals all showed a slight loss. At intervals approximately 0.2 cc. of blood was obtained from the tail by means of a small collecting capsule. Phosphatase activity and inorganic phosphate determinations were made on this blood by a microprocedure previously described (9). The values obtained by averaging the results of the phosphatase and inorganic phosphate determinations for each group are given in Table I.

TABLE II

Chemical Findings at Autopsy

Rat No.*	"Alkaline" phosphatase	Serum inorganic phosphate	Calcium	Total protein	Femur Ca:P	Remarks
	units	*mg. per cent*	*mg. per cent*	*per cent*		
H-131	110	4.4	10.0	6.57	1.92	Hypophysectomy complete
H-135	125	4.8	13.1	5.12	1.77	" "
H-138	130	4.0	10.3	5.75	1.65	" "
C-131	49	7.0	9.4	6.20	1.76	
C-135	78	8.3	9.7	6.15	1.87	

* H indicates the animal was hypophysectomized; C represents control.

Five animals (two control, three operated) were sacrificed 70 days after hypophysectomy. Blood chemistry studies included serum calcium (Tisdall (12)) and total protein (Kagen (4)). Completeness of hypophysectomy was confirmed by examination of

[1] The stock diet consisted of yellow corn-meal 67 per cent, linseed oil meal 12 per cent, casein with arginine 16 per cent, alfalfa meal 3 per cent, sodium chloride 1 per cent, and calcium carbonate 1 per cent.

the sella. The femur was analyzed for the Ca:P ratio. The data obtained are given in Table II.

DISCUSSION

The elevation of the alkaline phosphatase activity of eight hypophysectomized rats was definitely greater than that of six control litter mates. The study involved 111 determinations over a 10 week period. The individuals within both groups showed marked differences in phosphatase activity (Table I). This individual variation was also noted by Chen, Freeman, and Ivy (2) in a series of analyses of rat serum and tissue phosphatase activity. Since the controls were from the same litters as the hypophysectomized animals, comparison of the averages for each group was made. This comparison shows the average in the hypophysectomized group to be 20 to 96 per cent higher than that of the controls, which is a significant difference. The explanation of this difference will require additional investigation.

The rise of phosphatase activity in the control group may be related to growth. During the test period each of these animals gained approximately 70 gm. in weight. The rats in this investigation were observed from about the age of 70 days to 154 days. Weil (14) found a rise in plasma phosphatase activity during growth of normal rats from birth to 44 days.

The lowered inorganic phosphate of the hypophysectomized group confirms the reported findings of Hetherington and Weil (3) and Snyder and Tweedy (10). This reduced phosphate is clearly shown by the serial determinations. Here again individual differences are found as in the phosphatase activity. This wide range of values illustrates how some of the contradictory findings reported may have arisen (13). A difference in calcium was suggested by the five determinations done at autopsy. Serial determinations would be essential to follow the calcium and phosphate changes in relation to each other.

On gross examination the bones of the hypophysectomized groups were found to be smaller and thinner. This was very marked in the skull and has been described in detail by Mortimer (5). The Ca:P ratio, however, did not reveal any chemical difference between the two groups of animals (Table II).

SUMMARY

1. Eight hypophysectomized rats were found to have an elevation of phosphatase activity above that obtained for six control litter mates. This elevation persisted for at least 10 weeks after operation.

2. The inorganic phosphate level of the control animals remained consistently above that of the hypophysectomized group.

3. At the end of 10 weeks the serum calcium of three of the hypophysectomized animals was higher than that of two controls.

We are indebted to Dr. T. S. Sutton, Department of Animal Husbandry, for providing us with the animals used for this study and the stock diet fed during the experimental period, and to Dr. H. L. Reinhart, Pathologist of the University Hospital, for his criticisms and suggestions.

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STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

VII. THE EFFECT OF 3,3′-METHYLENEBIS(4-HYDROXYCOUMARIN) ON THE PROTHROMBIN TIME OF THE PLASMA OF VARIOUS ANIMALS*

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This communication deals with the effect of 3,3′-methylenebis-(4-hydroxycoumarin), the hemorrhagic and anticoagulant agent present in spoiled sweet clover hay, on the prothrombin time of the plasma of various animals (1–6). Detailed dosage responses for susceptible rabbits are given, as well as representative responses to single dosage levels for rats, guinea pigs, and dogs. The studies with susceptible rabbits and other animals have shown that the extent and duration of the hypoprothrombinemia, as reflected by the increase in the clotting time of 12.5 per cent plasma, are functions of the amount of 3,3′-methylenebis(4-hydroxycoumarin) fed.

In the bioassay developed for our work on the isolation and identification of the anticoagulant,† the extent of the diminution of the prothrombin level in the plasma of rabbit blood was expressed by the relative clotting index (2). This value is the ratio

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† The term anticoagulant is used in the general sense that 3,3′-methylenebis(4-hydroxycoumarin) is an agent which, after action *in vivo*, impairs or prevents the coagulation of blood. It does not affect the clotting power when added *in vitro* to blood or plasma ((2) p. 12, (4) p. 26).

of the concentration of the normal plasma, in the concentration range of 12.5 to 8.34 per cent, to the concentration of the pathic plasma,[1] in the range 25 to 8.34 per cent, which would give the same clotting time. Physiologically active hays or concentrates prepared therefrom give relative clotting indices of less than 1.0. The problem of biological variation in the assay rabbits was met by using only individually standardized susceptible rabbits whose relative clotting indices fell below 0.60 after they had eaten 50 gm. of the standard sample of spoiled sweet clover hay (2, 3). By comparing the pathic plasma with plasma from the same animal in the normal state, the assay was placed on a strictly differential basis. This eliminated the necessity of obtaining absolute prothrombin values.

After the 3,3′-methylenebis(4-hydroxycoumarin) became available in quantity, it was found that the feeding of more than 3.0 mg. in a single dose to our susceptible rabbits resulted in pathic plasmas whose clotting times, within the concentration range of 25 to 8.34 per cent plasma, could not be matched with the clotting time of normal plasma in the 12.5 to 8.34 per cent concentration range. Therefore the use of the relative clotting index as a means of expressing the responses reported in this study was abandoned in favor of simply indicating the clotting time of the pathic plasma at the 12.5 per cent concentration. The use of the relative clotting index is, however, advantageous in the standardization of susceptible rabbits, and for dosage studies in the lower ranges. The plasma concentration of 12.5 per cent (1 part of plasma, 7 parts of saline solution) was selected in part for reasons previously stated ((2) pp. 7–9) and for others which will follow in the discussion.

The prothrombin time obtained with susceptible rabbits is given for the range 0.37 to 6.0 mg. of the hemorrhagic agent. In the discussion, the mendelian situation encountered in the rabbit is considered in the light of the response of resistant rabbits to continued feeding of high levels of the 3,3′-methylenebis(4-hydroxycoumarin) and administration by injection. It is shown that rabbits classified as resistant on the basis of the standardiza-

[1] By pathic plasma we mean plasma in which the prothrombin level (or activity) has been altered from the normal by the hemorrhagic agent (see (2) p. 1).

tion procedure will respond to the 3,3′-methylenebis(4-hydroxycoumarin) when it is injected intravenously as the disodium salt.

Representative prothrombin times for one dosage level are given for the rat, guinea pig, and dog. The detectable dosage is indicated as well as the effect of single massive feedings.

It should be emphasized (*a*) that the prothrombin time of 12.5 per cent plasma will indicate the onset of the hypoprothrombinemia *before* changes in the prothrombin time of whole plasma are observed, (*b*) that the whole blood clotting times will usually be unaffected when the reduction in the prothrombin level or activity becomes detectable, and (*c*) that the hemorrhagic condition characteristic of the sweet clover disease does not appear unless the 3,3′-methylenebis(4-hydroxycoumarin) is fed or injected *continuously* over a period of time.

In the discussion there is considered among other things, our point of view on the vexing question of translating plasma clotting times into percentage prothrombin on the basis of the dilution curve principle. We have also indicated the general plan of our synthetical studies on the analogues of 3,3′-methylenebis(4-hydroxycoumarin) and its derivatives.

EXPERIMENTAL

Dosage Studies with Standardized Susceptible Rabbits—The method of standardizing the individual rabbits and determining the clotting time (prothrombin time) of the plasma has already been given in detail (2). The rabbits were in good physical condition, 2 to 3 years old, and weighed about 2.5 kilos. The selection of the individuals was made on the basis of uniformity of response to 50 gm. of the standard spoiled hay sample, and to 2.5 mg. of the pure 3,3′-methylenebis(4-hydroxycoumarin) (relative clotting indices 0.50 to 0.30).

Extensive preliminary studies indicated that when less than 0.38 mg. of the hemorrhagic agent was fed, a demonstrable increase in the clotting time could not be established. Quantities above 6.0 mg. gave more prolonged clotting times, but there was a much greater variation in the response of the individual animals. Therefore, levels up to 6.0 mg. were fed to each rabbit and the in-

crease in clotting time of the 12.5 per cent plasma was measured at definite intervals after feeding until the normal clotting time was restored. An 8 to 10 day rest period was allowed after the restoration of normal clotting times.

The 3,3′-methylenebis(4-hydroxycoumarin) was prepared for oral administration by weighing directly into hard gelatin capsules. The anticoagulant was fed at the levels of 0.37, 0.75, 1.5, 3.0, and 6.0 mg. Each level was fed to six rabbits and each rabbit

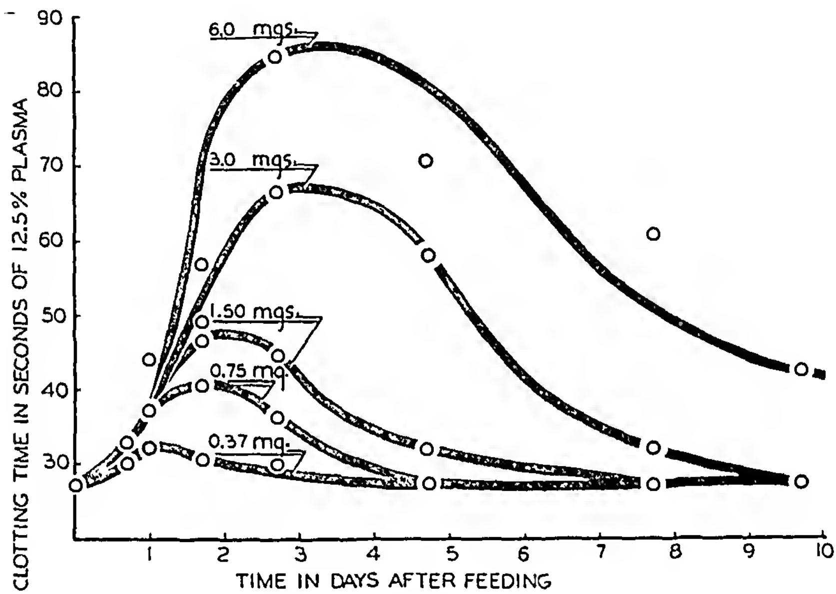


FIG. 1. The effect of feeding different levels of 3,3′-methylenebis(4-hydroxycoumarin) on the prothrombin time of susceptible rabbits (average curves for six animals).

received all levels. The order of feeding the levels was arranged so that a possible effect by the previous feedings could be detected, but no such effect was observed.

The rabbits were given only water for 24 to 36 hours before administration of the dose and only water thereafter until the prothrombin times were normal. Blood samples were drawn at regular intervals and the prothrombin times determined. The average prothrombin time of the group for each dosage level is indicated in Fig. 1.

Dosage Studies with Other Animals—Although we have accumu-

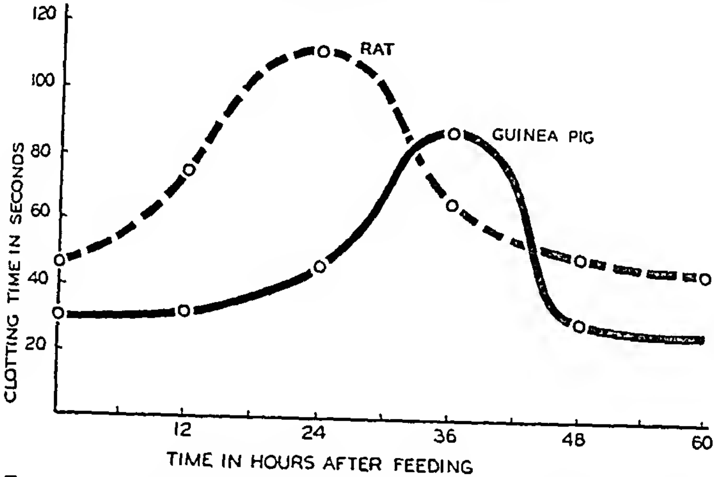


Fig. 2. The effect of feeding 3,3′-methylenebis(4-hydroxycoumarin) on the prothrombin time of rats and guinea pigs. Average curve of the clotting time of 12.5 per cent plasma for rats fed 2.5 mg. and of whole plasma from guinea pigs fed 10.0 mg.

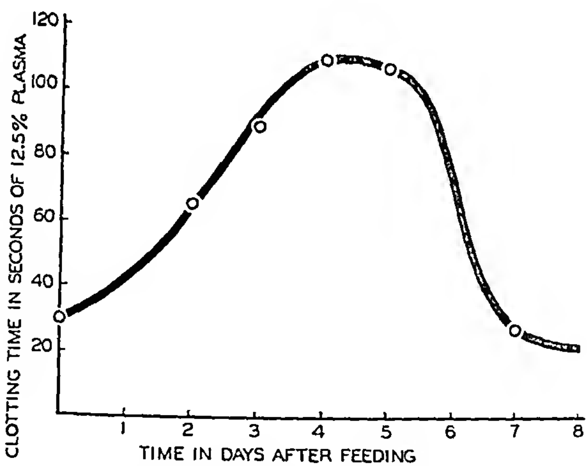


Fig. 3. The effect of feeding 50 mg. of 3,3′-methylenebis(4-hydroxycoumarin) on the prothrombin time of 5 kilo dogs.

lated many data on the response of rats, guinea pigs, and dogs to 3,3′-methylenebis(4-hydroxycoumarin), for the purpose of this communication it will suffice to record the response to a single

level for each species.[2] The responses of the rat to 2.5 mg. and the guinea pig to 10 mg. are given in Fig. 2, and the response of dogs to 50 mg. (oral administration) is given in Fig. 3. The rats were fasted 12 hours prior to feeding the anticoagulant mixed in the standard ration, but access to the stock ration was allowed thereafter. The guinea pigs[3] and dogs were fasted 24 hours, then fed the anticoagulant in capsules, and given access to their respective standard rations. The blood samples from the rats and guinea pigs were taken by heart puncture, while those from the dogs were obtained by venipuncture.

DISCUSSION

Response of Susceptible Rabbits—Fig. 1 shows that, when 3,3'-methylenebis(4-hydroxycoumarin) is fed to standardized susceptible rabbits with approximately the same relative clotting indices, the increase in clotting time (extent of the hypoprothrombinemia) and the period required for the restoration of normal clotting values are functions of the dosage level.

The average normal clotting time of the 12.5 per cent plasma of the six rabbits was 27 seconds with a standard deviation of the mean of 1.0 second. The average maximum clotting time resulting from the administration of 0.37 mg. of the anticoagulant was 32 seconds with a deviation of 1.7 seconds. The 6.0 mg. level produced an average maximum clotting time of 84 seconds, with a deviation of 12 seconds. Within the range of 0.37 to 6.0 mg., the maximum increase in clotting time is almost proportional to the dosage level. Although dosage levels above 6.0 mg. give slightly higher maximum clotting times, the increase is not proportional to the increase in dosage.

The prothrombin time of one of the animals given 6.0 mg. of

[2] The relationship between clotting time of normal and pathic plasma of 12.5 per cent concentration after feeding the 3,3'-methylenebis(4-hydroxycoumarin) to the rabbit, rat, and dog under our conditions are the following: normal rabbit plasma 25 to 35 seconds, pathic after feeding 2.5 mg. 60 to 70 seconds; normal rat plasma 35 to 45 seconds, after feeding 2.5 mg. 90 to 115 seconds; normal dog plasma 25 to 35 seconds, after feeding 50 mg. 90 to 110 seconds.

[3] The thromboplastin used was prepared from the brain of the guinea pig by the method given previously for rabbit brain (2).

the anticoagulant was normal 3 days after administration, while the normal clotting time in another was not restored until the 11th day. The other animals showed normal clotting times between these time limits.

This wide variation in the maximum clotting times and in the period required for the restoration of normal values holds only for the high dosage levels. At the lower levels all the animals returned to normal in approximately the same time. It would appear that the individual variation between the test animals exhibited at the higher dosage levels is due in part to variations in absorption of the anticoagulant.

Response of Resistant Rabbits—It has been indicated previously that the marked variation in the susceptibility of some rabbits to the hemorrhagic agent is inherited as a mendelian character and that the resistance exhibited by other individuals is not an absolute but a relative resistance ((2) p. 15, (3)). The following experiments will serve to illustrate this point. A group of rabbits classified as resistant on the basis of our standardization procedure showed no response to four successive daily feedings of 10 mg. of the hemorrhagic agent. They were then fed a ration of U. S. No. 1 Extra Leafy Extra Green alfalfa hay and the stock grain mixture *ad libitum*. 1 mg. of the crystalline 3,3′-methylenebis(4-hydroxycoumarin) was given daily via capsule for 30 days. Prothrombin times were determined at 3 day intervals on the 12.5 per cent plasma. No hypoprothrombinemia developed. 10 mg. of the anticoagulant were then fed each day for 14 days. No response was observed. Then the dosage level was increased to 25 mg. per day for 10 days and again no response was noted.[4] Finally 50 mg. of the 3,3′-methylenebis(4-hydroxycoumarin) were fed to some of the rabbits and in others 10 mg. of the disodium salt were injected intravenously. Hypoprothrombinemia was then observed in some of the rabbits fed the 50 mg. dose and in *all* of the rabbits that received the anticoagulant by injection (Fig. 4). It is therefore quite probable that hypoprothrombinemia can be induced in any rabbit, irrespective of the resistance shown with

[4] The addition of bile salts to facilitate absorption in these trials was without effect.

oral administration, if the 3,3'-methylenebis(4-hydroxycoumarin) be injected in sufficiently large doses.[5]

It appears that the tremendous variation between individual rabbits in their response to oral administration of 3,3'-methylenebis(4-hydroxycoumarin) is, at least in part, associated with a variation in their ability to absorb the substance from the digestive tract.[6] Forthcoming communications will deal with the influence of *l*-ascorbic acid, and certain antihemorrhagic agents on the

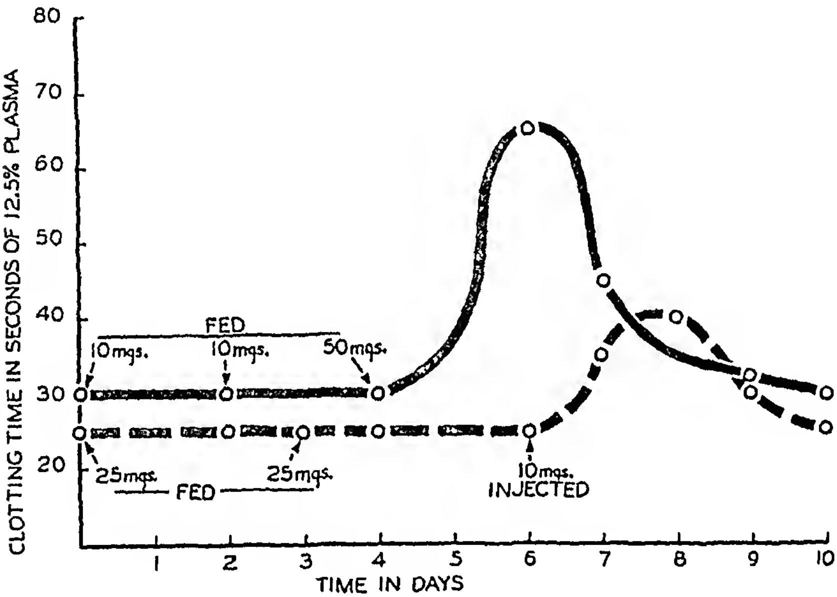


FIG. 4. The effect of feeding and injecting 3,3'-methylenebis(4-hydroxycoumarin) on the prothrombin time of resistant rabbits.

anticoagulant action of 3,3'-methylenebis(4-hydroxycoumarin) in the rabbit and the rat.

Response of Rats and Guinea Pigs—In contrast to the rabbit, no indications of relative resistance to the prothrombin-inactivat-

[5] Solutions for injection are prepared by dissolving 10.0 gm. of 3,3'-methylenebis(4-hydroxycoumarin) in 1.0 liter of water containing 2.86 gm. of sodium hydroxide. After the anticoagulant has dissolved, 1.3 gm. of sodium sulfite are added to stabilize the solution. The final pH of such solutions will be between 10.5 and 11.5. Sterilization by filtration through a Seitz E. K. filter and storage in sealed ampules at 5° are recommended.

[6] It is recorded that the horse does not develop the hemorrhagic sweet clover disease from the eating of spoiled sweet clover hay (7).

ing properties of the 3,3′-methylenebis(4-hydroxycoumarin) have been observed in rats, guinea pigs, and dogs. All of the rats used to date (over 400) have responded to a single feeding of 2.5 mg.[7] The detectable dose is about 1.0 mg. 10 mg. fed to rats on each of 3 consecutive days caused a drastic prolongation in the clotting time, but the animals survived. In the rat the hypoprothrombinemia attains a maximum more quickly (24 hours instead of 72) after a single dose, and is of shorter duration than in the rabbit. Guinea pigs show a clotting time curve similar to that of the rat. On a mg. per kilo of body weight basis the rat is much less sensitive to the 3,3′-methylenebis(4-hydroxycoumarin) than the rabbit.

Response of Dog to 3,3′-Methylenebis(4-hydroxycoumarin)—The increase in the clotting time produced by feeding the hemorrhagic agent to dogs is quite similar to that realized with the rabbit in that the effect of a single dose is prolonged over a period of days (Figs. 1 and 3).[8] Variations in the response of different dogs to a given dose have been observed, but up to the present we have not had the time and facilities to conduct extensive dosage studies comparable to those made with susceptible rabbits. With 8 to 10 kilo dogs the effect of one 10 mg. dose (by mouth) is detectable by our method of assay. 10, 25, and 50 mg. have been fed daily for 10 days to 2 weeks with no detectable effects except the hypoprothrombinemia. The responses are essentially the same in fasting and in fed animals. All dogs tested to date have survived *single* massive feedings (1.0 gm. per kilo of body weight). Absorption of 3,3′-methylenebis(4-hydroxycoumarin) from the tract of the dog seems to be incomplete. Two 6 kilo dogs were fed 200 mg. doses every 12 hours along with a standard diet until they succumbed on the 6th day. Their feces and urine were collected during this period. From the combined feces 0.58 gm. of 3,3′-methylenebis(4-hydroxycoumarin), m.p. 288–289°, was recovered by a procedure based in principle on the method of extraction used

[7] Based, in the main, on studies dealing with the response of the rat to 3,3′-methylenebis(4-hydroxycoumarin), conducted jointly with our colleagues Dr. Carl Baumann and Mr. J. B. Field, which will appear later.

[8] Confirmed by Dr. O. O. Meyer and Dr. J. B. Bingham at the Wisconsin General Hospital and by the work of Dr. H. R. Butt and associates at the Mayo Clinic.

in the isolation work on spoiled sweet clover hay (1–3). The quantity of 3,3′-methylenebis(4-hydroxycoumarin) recovered is equivalent to approximately 15 per cent of the amount fed. The urine was likewise investigated, but was found to be free from the 3,3′-methylenebis(4-hydroxycoumarin) both on the basis of the isolation technique and the bioassay (2). This observation, in conjunction with the tolerance of individual animals to massive single feedings of 3,3′-methylenebis(4-hydroxycoumarin) and the greatly increased effect when equivalent amounts are administered by injection, indicates that in the dog absorption of this material from the tract is not complete. Studies on the fate of the 3,3′-methylenebis(4-hydroxycoumarin) in the animal body are in progress.

The characteristic lesions as seen in the hemorrhagic sweet clover disease of ruminants (7) were induced in these dogs.[9]

3,3′-Methylenebis(4-hydroxycoumarin) and Percentage of Prothrombin Depletion Estimated by Dilution Curve Principle—Quick (8) has estimated the percentage of prothrombin by determining from a dilution curve the concentration of recalcified normal plasma which has the same clotting time as the whole pathic plasma (see also (2) Figs. 1 and 2, pp. 8–10). The concentration of normal plasma is considered to be equivalent to the percentage prothrombin of the plasma being appraised.

Fig. 5 shows the average clotting time of oxalated plasmas when serially diluted with saline solution with the addition of thromboplastin and calcium chloride. The normal curve represents an average of normal rabbit plasmas, while the pathic curve represents an average of plasmas obtained 72 hours after feeding 6.0 mg. of 3,3′-methylenebis(4-hydroxycoumarin). It is evident from Fig. 5 that the dilution curve of the pathic plasmas differs from the normal in two respects. The whole plasma clotting time has been greatly increased and less dilution is required to effect a marked (corresponding) increase in the clotting time. The extent of the aforementioned changes in the pathic plasma is dependent on the quantity of 3,3′-methylenebis(4-hydroxycoumarin) fed. However, it is to be noted that the curve obtained by diluting the

[9] We are indebted to our colleague, Mr. H. B. Parry, M.R.C.V.S., Commonwealth Scholar from Cambridge, England, for making the postmortem examinations on these animals.

pathic plasmas is not superimposable on the normal plasma curve by a shift of the axis.

The latter fact, along with the previously mentioned instability of the pathic plasmas (rapid inactivation on standing and greater heat lability ((2) p. 11)), makes it appear plausible that the effect of the action of the 3,3′-methylenebis(4-hydroxycoumarin) is

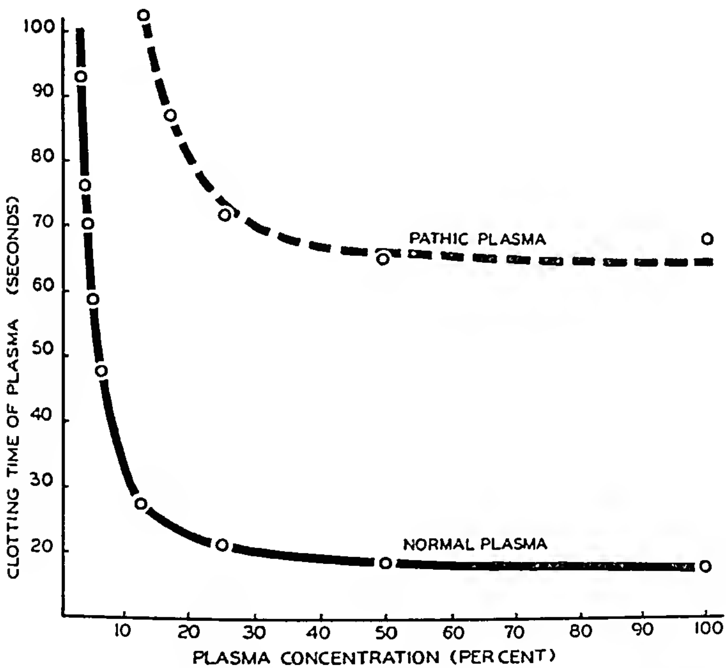


FIG. 5. The effect of dilution on the prothrombin time of normal rabbit plasma and of pathic plasma obtained 72 hours after feeding 6.0 mg. of 3,3′-methylenebis(4-hydroxycoumarin).

perhaps not restricted to a disturbance in the integrity of the prothrombin mechanism.

When the plasma dilution curve principle was applied to the estimation of the prothrombin content of the pathic plasmas, it was found that the concentration of the plasma on which the clotting time was taken affected the results. If the prothrombin content of whole pathic plasma is estimated by referring to the

dilution curve of the normal plasma, a much lower value (percentage) is obtained than if the estimation is made from the clotting time of the diluted pathic plasma. This can be illustrated by the following example.

The average clotting time of the undiluted plasma of susceptible rabbits 72 hours after feeding 6.0 mg. of 3,3′-methylenebis(4-hydroxycoumarin) was 60 seconds. Since this corresponds to the clotting time of a normal plasma at 5 per cent concentration, the prothrombin level would be estimated at 5 per cent of normal. However, with the 12.5 per cent plasma as the standard of comparison,[10] the clotting time of the diluted pathic plasma, at 80 seconds, corresponds to the clotting time of a 33 per cent concentration of the 12.5 per cent normal plasma (4 per cent concentration of whole plasma). Therefore, on the basis of diluted plasmas, the prothrombin would be estimated at 33 per cent of normal.

In view of this difficulty in translating plasma clotting times into per cent prothrombin, we have chosen to express the results presented in this communication by the clotting time of recalcified 12.5 per cent plasma. It would appear that the plasma dilution at which the final clotting time is measured will affect the value of any method based on the clotting time of normal diluted plasma.

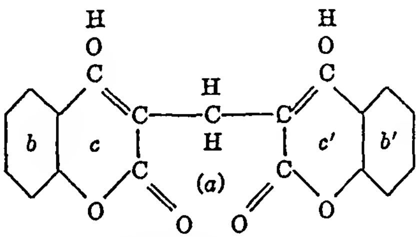
The point at which hemorrhage begins in the various hypoprothrombinemias, as judged by the plasma prothrombin time, is a matter of much debate (8–14). We prefer not to become involved in the controversy and would like to call to the attention of the prothrombin specialists the following. It has been observed that dogs with a whole plasma prothrombin time of approximately 10 minutes induced by a *single* large dose of 3,3′-methylenebis(4-hydroxycoumarin) showed no tendency to hemorrhage.[11] The same observation has been made on rats and rabbits having prothrombin times in excess of 250 seconds (12.5 per cent plasma). The fact that hemorrhage does not accompany these drastic reductions in the prothrombin levels resulting from a single dose

[10] Assigning to the prothrombin content of 12.5 per cent plasma the value of 100 per cent of normal.

[11] This observation has been substantiated by Dr. H. R. Butt and associates (personal communication to K. P. L., May 27, 1941), "A dog given 100 mg. per day (oral) showed a whole plasma prothrombin clotting time of 35–45 minutes, the prothrombin percentage being nearly zero."

suggests that the onset of the bleeding tendency in the hemorrhagic sweet clover disease involves more than a simple suspension of normal prothrombin levels or activity.[12]

Relation of Structure in the 3,3'-Methylenebis(4-hydroxycoumarin) Group to Physiological Activity—Since April, 1940, we have been cognizant of the fact that the capacity to induce the hypoprothrombinemia which characterizes the hemorrhagic sweet clover disease is not restricted to the compound found in nature. In view of the structure of 3,3'-methylenebis(4-hydroxycoumarin) opportunity is afforded to synthesize analogues wherein substitution has been effected on (1) the methylene carbon atom, *a*; (2) the benzene rings, *b*, *b'*; and (3) esterification of the hydroxyl group on the α-pyrone rings, *c*, *c'*. In addition our studies have



3,3'-Methylenebis(4-hydroxycoumarin)

included various 3-substituted 4-hydroxycoumarins, as well as degradation products obtained from 3,3'-methylenebis(4-hydroxycoumarin) and 4-hydroxycoumarin.

Since June, 1940, a squad of workers headed by Dr. Mark A. Stahmann (Mr. C. F. Huebner, Mr. W. R. Sullivan, Mr. R. S. Overman, Mr. D. G. Doherty, Dr. I. A. Wolff, and Dr. S. A. Karjala) has prepared more than 150 compounds, many of them analogues of the parent product (*vide ut supra*), and others representing structures in part related.

This study has already led to over forty compounds with prothrombin-reducing properties (susceptible rabbits as the test animals). In view of the pitfalls that are inherent in the pre-

[12] Preliminary studies on the action of 3,3'-methylenebis(4-hydroxycoumarin) in man have been reported by Bingham, Meyer, and Pohle (15) and Butt, Allen, and Bollman (16).

mature publication of any study dealing with the relationship of molecular structure to physiological activity, and to avoid lost motion, we shall be glad to disclose to those interested the present status of our work and the plans for the immediate future, since publication will be withheld until the activity of each compound has been established by repeated testing at several dosage levels.

SUMMARY

1. The response of susceptible rabbits to the feeding of 3,3′-methylenebis(4-hydroxycoumarin) for the range 0.37 to 6.0 mg., as measured by the increase in the clotting time of 12.5 per cent plasma, and the period of prolonged coagulation are given.

2. Representative responses of rats, guinea pigs, and dogs to single dosage levels are presented.

3. It is shown that rabbits classified as resistant to 3,3′-methylenebis(4-hydroxycoumarin) on the basis of limited oral feeding develop hypoprothrombinemia when the substance is injected intravenously or fed in large doses.

4. The clotting time of progressively diluted normal plasma and similarly diluted plasma obtained after the administration of 3,3′-methylenebis(4-hydroxycoumarin) is discussed in relation to the extent of prothrombin depletion.

5. The hypoprothrombinemia caused by 3,3′-methylenebis(4-hydroxycoumarin) can also be induced by some of its analogues and derivatives.

A special acknowledgment is due my colleague and former pupil, Dr. R. J. Dimler, whose critical faculty and objective outlook have been of the greatest service in conjunction with our studies on the hemorrhagic sweet clover disease (K. P. L.).

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A SUPPLEMENTARY GROWTH FACTOR FOR LACTOBACILLUS CASEI*

Sirs:

In routine assays for pantothenic acid by the method of Pennington *et al.*,[1] it was found that the slope, *b*, of the dose-response curve tended to be greater when rice polishings concentrate[2] was assayed than when pure calcium pantothenate was used.

Quantities of the rice polishings concentrate larger than those usually used in an assay promoted acid production by *Lactobacillus casei* well beyond the amounts formerly considered maximum; *i.e.*, between 9 and 10 ml. of 0.1 N acid in 72 hours of incubation. The maximum titrations obtained were 20 to 23 ml. with 400 mg. or more of rice polishings concentrate per 10 ml. of medium. The effect of increased acid production was not removed by one treatment of the concentrate with fullers' earth at pH 4. The sugar content of the preparation accounts for only a small fraction, if any, of the increased acid production. The factor we are concerned with seems to be present in much smaller concentration in two commercial yeast concentrates; it could not be demonstrated in yeast extract (Difco, Bacto) which has been used

TABLE I

Substance tested	No. of cases	Mean slope, *b**	Standard error of mean
Calcium pantothenate	8	8.04	±0.28
Rice polishings concentrate	23	9.17	±0.15

* *b* is the slope of a straight line relating the logarithm of the dose and ml. of 0.1 N acid produced in 72 hours of incubation. The difference between mean slopes is significant (t = 3.655 and P is <0.01).

* The authors wish to acknowledge the cooperation and advice of Dr. L. Reiner, Scientific Director of the Burroughs Wellcome and Company, U. S. A., Experimental Research Laboratories.

[1] Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **135**, 213 (1940).

[2] Ryzamin-B, Burroughs Wellcome and Company (U. S. A.), Inc.

as a component of the basal medium and as a source of growth factors for *Lactobacillus casei*.[3]

When rice polishings concentrate is autoclaved in NaOH at pH 12, 15 pounds, for 30 minutes, the supplementary growth factor is destroyed. Under these conditions the factor appears to be more labile than the pantothenic acid present in the concentrate. Only about one-half of the supplementary effect is removed by incubation overnight at 50° with taka-diastase. This fact

TABLE II

Substance tested	Amount in 10 ml. medium	0.1 N acid produced in 72 hrs.
	mg.	*ml.*
Rice polishings concentrate	24	10.4
" " "	96	12.2
" " "	384	19.8
" " " treated with fullers' earth	384	21.1
Dextrose, levulose, and sucrose	100 each	11.9
+ Calcium pantothenate	0.1	
Calcium pantothenate controls	0.1	9.2
Rice polishings concentrate, autoclaved	192	9.3
" " " "	192	9.2
+ Calcium pantothenate	0.1	
Rice polishings concentrate, treated with 100 mg. taka-diastase	400	15.8
Rice polishings concentrate, untreated	400	20.9

as well as the absence of any increased acid production when wheat flour was tested suggests that the substance in question is different from that mentioned by Scott *et al.*[4]

Further work is being directed toward characterization and separation of the factor supposed to be responsible for the enhanced acid production.

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MARIE LECHYCKA
AMOS E. LIGHT

Received for publication, December 30, 1941

[3] Snell, E. E., and Peterson, W. H., *J. Bact.*, 39, 273 (1940).
[4] Scott, M. L., Randall, F. E., and Hessel, F. H., *J. Biol. Chem.*, 141, 325 (1941).

CRYSTALLINE PREPARATION OF CARBONIC ANHYDRASE

Sirs:

Reports have recently been made concerning the purification[1] and certain properties of carbonic anhydrase.[2] Further attempts to purify the enzyme preparation by means of inorganic salts or with various solvents met with little success and it therefore seemed possible that the product had a degree of purity approaching that of a pure substance. Attempts to crystallize the enzyme by means of the various inorganic salts commonly used in the crystallization of enzymes were unsuccessful. Accordingly, other means were sought. The enzyme preparation was unstable in 40 per cent ethyl alcohol and insoluble in 60 per cent ethyl alcohol. It was thought that this instability and insolubility in alcohol might be due to a small amount of inorganic salts in the enzyme preparation. Attempts were made to reduce the content of inorganic material in the following manner.

To a concentrated solution of enzyme a small amount of dipotassium hydrogen phosphate was added, followed by the addition of alcohol to a concentration of 30 per cent. This solution was thoroughly shaken with chloroform and placed in the refrigerator overnight. During this time a small amount of foreign protein separated. The mixture was centrifuged and the supernatant alcoholic solution of enzyme again shaken with chloroform. After standing overnight the mixture was centrifuged. The alcoholic enzyme solution was removed and dialyzed for 48 hours against distilled water. The aqueous enzyme solution thus obtained was dried *in vacuo*. The dried material contained approximately 10,000 units of enzyme per mg. This enzyme preparation was completely soluble in 99 per cent ethyl alcohol and in such a solution was stable for 24 hours at room temperature. To crystallize the enzyme from this alcoholic solution ether was added to 33 per cent concentration, producing a faint opalescence, and dry

[1] Scott, D. A., and Mendive, J. R., *J. Biol. Chem.*, 139, 661 (1941).

[2] Scott, D. A., and Mendive, J. R., *J. Biol. Chem.*, 140, 445 (1941).

ammonia gas was bubbled through the solution until saturation with ammonia was attained. These operations were carried out in an ice bath. Within 5 hours 80 per cent of the enzyme activity was found in the crystalline precipitate which had settled to the bottom of the liquid. On standing overnight, over 90 per cent yields of crystalline carbonic anhydrase have been regularly obtained. Examined under the microscope these precipitates appear to be completely crystalline. The crystals are thin plates which when standing on their edge present a needle-like appearance. Attempts to dry the crystals without causing a destruction of enzyme activity have thus far failed.

Experiments are in progress to determine what part, if any, zinc may play in the crystallization. Other chemical and physical properties of the crystalline enzyme are being determined.

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D. A. Scott

Received for publication, December 8, 1941

THE CONCENTRATION OF THE SUBSTANCE IN BLOOD WHICH IS STIMULATORY FOR LACTOBACILLUS CASEI ϵ

Sirs:

In a recent publication[1] it was reported that the recovery of riboflavin added to certain mammalian bloods as determined by the Snell and Strong microbiological method[2] was somewhat too high. It was later shown that alkaline photolyzed human blood also gave high recoveries in the riboflavin assay,[3] and that alkaline autoclaved suspensions of fresh liver gave anomalous results when assayed for pantothenic acid by the microbiological method.[4] Furthermore, various fat-soluble compounds have been found to stimulate *Lactobacillus casei* ϵ, and ether extraction has been recommended for samples which are to be subjected to microbiological assay for riboflavin or pantothenic acid.[5]

Fat-soluble preparations which simulate the stimulatory action of blood have now been obtained from beef and dog bloods by continuous ether extraction. Further purification was accomplished by partitioning between ether and water and between dilute alcohol and petroleum ether. Typical preparations showed marked stimulation at a level of approximately 75 γ per 10 cc. of medium. As estimated from the stimulatory effect of whole blood and of photolyzed blood the active material was concentrated 500 to 1000 times. A large part of the original activity was retained in the concentrates.

[1] Strong, F. M., Feeney, R. E., Moore, B., and Parsons, H. T., *J. Biol. Chem.*, **137**, 363 (1941).

[2] Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 346 (1939).

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[5] Bauernfeind, J. C., Sotier, A. L., and Barreff, C. S., Atlantic City meeting of the American Chemical Society, Abstracts, Division of Biological Chemistry, p. 34, September, 1941.

The degree of stimulation was estimated by determining the apparent riboflavin by the Snell-Strong procedure when 0.1 γ of riboflavin and varying amounts of the above preparations were added to the assay tubes. Recoveries of 200 per cent and over have been observed. An even greater stimulation was found in the assay for pantothenic acid. Two levels of a particular preparation gave riboflavin recoveries of 135 and 187 per cent, and pantothenic acid recoveries of 235 and 255 per cent. Several fold higher levels caused inhibition.

The active material was stable to autoclaving for 20 minutes in 0.5 N alkali or in 0.5 N acid, but was destroyed by autoclaving for 12 hours in 26 per cent hydrochloric acid. It was soluble in ether, petroleum ether, and alcohol, and was removed from blood by continuous ether extractions at pH 1.5, 7.3, or 10. It appeared in the filtrate after the blood proteins were precipitated with 80 per cent alcohol. No crystalline preparations have been obtained.

The stimulatory material appeared to be absent from or present in low concentrations or in an inactive form in liver, yeast, milk, and urine. However, an alcoholic extract of whole fresh liver when hydrolyzed with alkali was strongly inhibitory. The nature of this active material and its possible relationship to the blood stimulant are being investigated.

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INOSITOL, A CONSTITUENT OF A BRAIN PHOSPHATIDE

Sirs:

By the use of a recently developed method[1] it was found that phosphatides both from brain and spinal cord contained inositol. Brain cephalin[2] was found to be especially rich in it. When brain cephalin was fractionated by precipitation from $CHCl_3$ solution by increasing concentrations of alcohol,[3] followed by dialysis of the fractions, inositol was found only in the fraction least soluble in alcohol. Both phosphatidyl serine[4] and the fraction most soluble in alcohol were practically free of it. This evidence pointed to the existence of a new phosphatide in which inositol was a constituent. Inositol-containing phosphatides have been reported in tubercle bacilli[5] and in soy beans,[6] but have not been found previously in animal tissues.

The following facts showed that inositol was chemically combined in the lipid: (1) By microbiological assay the intact lipid was only one-twentieth as active as its hydrolytic products; (2) added inositol could be removed by dialysis, leaving the inositol content of the phosphatide unchanged; and (3) reaction of HIO_4[7] with the lipid showed no free inositol.

The phosphatide fraction obtained by the methods indicated above was a friable white powder containing 4.5 per cent P and about 1 per cent N, all of it amino N. Various preparations contained from 6.8 to 8.6 per cent inositol. It represented about one-fourth of brain cephalin, or 0.4 per cent of the net weight of the brain. By other methods preparations have been obtained which contained up to 10 per cent inositol.

[1] Woolley, D. W., *J. Biol. Chem.*, **140**, 453 (1941).
[2] Thudichum, J. L. W., A treatise on the chemical constitution of the brain, London (1884).
[3] Folch, J., *Proc. Am. Soc. Biol. Chem.*, in press (1942).
[4] Folch, J., *J. Biol. Chem.*, 139, 973 (1941).
[5] Anderson, R. J., *J. Am. Chem. Soc.*, 52, 1607 (1930).
[6] Klenk, E., and Sakai, R., *Z. physiol. Chem.*, 258, 33 (1939).
[7] Malaprade, L., *Bull. Soc. chim.*, **1**, 833 (1934).

Finally inositol was isolated by the following procedure. An aqueous emulsion of 3.5 gm. of the fraction (containing 6.8 per cent inositol) was precipitated by adding HCl up to 3 N concentration and the washed precipitate was hydrolyzed for 48 hours with boiling 6 N HCl. The hydrolysate was filtered and the filtrate treated with Ag_2O and H_2S in succession and finally concentrated and treated with 6 volumes of alcohol. Crystals formed which were collected after 10 days and recrystallized once from alcoholic HCl and once from alcohol, and finally dried at 100° in a vacuum. The yield was 140 mg.; m.p. 217.6° (uncorrected); mixed m.p. with inositol, 217.6° (uncorrected). Found, C 39.98, H 6.80 (corrected for 2.1 per cent ash). The hexaacetate was prepared; m.p. 211–213° (uncorrected); mixed m.p. with inositol hexaacetate, 211–213° (uncorrected). By microbiological assay the crystals were 100 per cent *meso*inositol within the limit of error of the method.

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